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Terpenoids

Part IV. The Structure of Occidol, a New Sesquiterpene Alcohol from *Thuja occidentalis* L.

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Department of Forest Products, Faculty of Agriculture, University of Tokyo

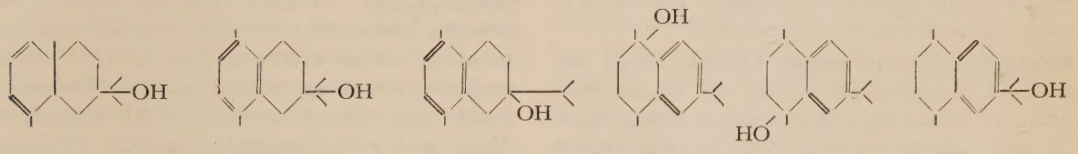
Received August 5, 1958

A new sesquiterpene alcohol, $C_{15}H_{22}O$, m.p. 69–70°C, named occidol, has been isolated from the essential oil of the wood of *Thuja occidentalis* L. The structure (II) is proposed for occidol.

In the previous paper[1] we have reported on the structure of occidentalol (I), $C_{15}H_{24}O$, a main component of the essential oil from *Thuja occidentalis* L. Besides this, we described the presence of another sesquiterpene alcohol, m.p. 69–70°C, having a higher boiling point than that of occidentalol, in the same essential oil.

On dehydrogenation with palladium charcoal, it affords an alkyl naphthalene, $C_{15}H_{18}$, in a good yield, which is supposed to be 1,4-dimethyl-6-isopropylnaphthalene by the melting points of its picrate and trinitrobenzene adduct.

The structure of this alcohol is therefore, represented by either (II), (III), (IV), (V) or (VI).



(I) (II) (III) (IV) (V) (VI)

This paper deals with the constitution of the sesquiterpene alcohol.

This sesquiterpene alcohol, $C_{15}H_{22}O$, m.p, 69–70°C, $[\alpha]_D^{25}+163.7°$, named occidol, has been isolated from the fraction, b.p. 135–145°C/3 mm, of the neutral portion. 3,5-Dinitrobenzoate melts at 166–167°C. The hydroxyl group is presumed tertiary since it does not react with phthalic anhydride and is readily eliminated with formic acid.

From the ultraviolet absorption maximum at 266 mμ ($\log \varepsilon$ 2.3) and the infrared absorption bands at 1605 and 805 cm^{-1}, it is evident that occidol contains an aromatic nucleous in its molecule. On catalytic hydrogenation with palladium charcoal, no hydrogen is absorbed. It is therefore, considered to be a dicyclic alcohol.

On ozonolysis of the hydrocarbon prepared from occidol by dehydration with formic acid, acetone and formaldehyde are obtained. On oxidation of occidol with alkaline potassium permanganate, followed by esterification with diazomethane, it yields tetramethyl benzene-1, 2, 3, 4-tetracarboxylate. Only formula (II) is in accord with the above experimental facts.

EXPERIMENTAL

Isolation of occidol The fraction, b.p. 135–145°C/3 mm of the neutral portion of the essential oil was difficult to crystallize, but on standing at room temperature for a long period it deposited a crystalline mass. The crystals were recrystallized from petroleum ether and aqueous methanol, m.p. 69–70°C, $[\alpha]_D^{25}+163.7°$ (c=1.22, in $CHCl_3$). *Anal.* Found: C, 82.49; H, 10.43. M.W. 229 (Rast method). Calcd. for $C_{15}H_{22}O$: C,

1) T. Nakatsuka and Y. Hirose, This Bulletin, **20**, 215 (1956).

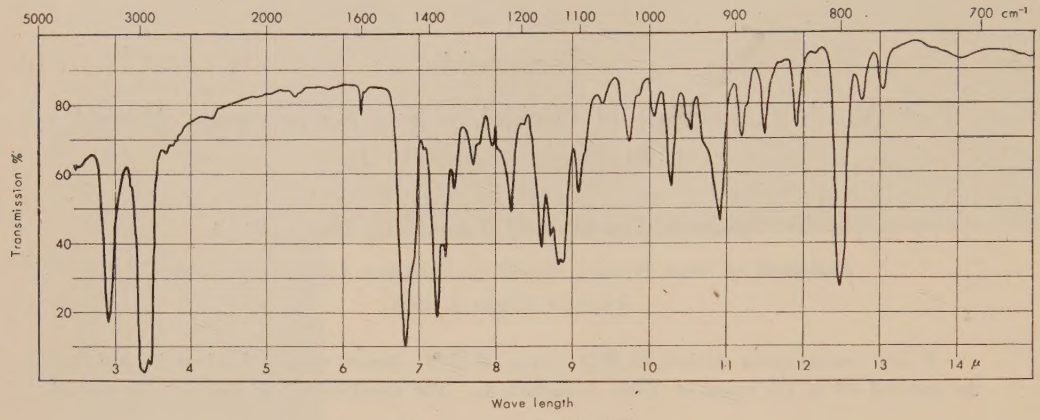


FIG. 1. Occidol (nujol)

82.51; H, 10.16%. M.W. 218. The ultraviolet absorption spectrum: λ_{max} 266 mμ, log ε 2.3. The infrared absorption spectrum is shown in Fig. 1. 3,5-Dinitrobenzoate prepared by the usual way melted at 166–167°C. *Anal.* Found: C, 63.58; H, 5.86; N, 7.16. Calcd. for $C_{22}H_{24}O_6N_2$: C, 64.06; H, 5.87; N, 6.79%.

Hydrogenation of occidol Occidol was shaken with hydrogen in the presence of palladium charcoal as a catalyst, but no hydrogen was absorbed.

Dehydrogenation of occidol Occidol (500 mg) was heated with 5% palladium charcoal for 5 hr. at 320–340°C. The ether-soluble product was chromatographed on alumina; elution with petroleum ether gave a colorless liquid, which afforded picrate, m.p. 101–103°C and a trinitrobenzene adduct, m.p. 141–144°C. *Anal.* of picrate. Found: C, 59.36; H, 4.77; N, 9.80. Calcd. for $C_{21}H_{21}O_7N_3$: C, 59.01; H, 4.95; N, 9.83%.

Dehydration of occidol and ozonolysis of the dehydrated product Occidol (800 mg) was heated with 90% formic acid (4 g) on a water bath for 1 hr. The product (600 mg) was distilled in vacuo, b.p. 140–150°C (oil bath)/3 mm. The infrared spectrum showed bands at 1600 (aromatic C=C), 806 (1,2,3,4-tetra-substituted benzene), 1639 (C=C) and 895 cm^{-1}(CR_1R_2 =CH_2), and hydroxyl absorption in the infrared spectrum disappeared.

The hydrocarbon (500 mg) described above in ethyl acetate (20 ml) was treated with ozone for 2 hr. The ozonide obtained by evaporation of the solvent under reduced pressure was decomposed with water and then steam-distilled into aqueous dimedone solution. The formaldehyde dimedone derivative, m.p. 189–190°C, thus obtained was identified by m.p. and mixed m.p. The filtrate from which the formaldehyde dimedone derivative was filtered off, was again steam-distilled into a solution of 2,4-dinitrophenylhydrazinesulfuric acid in aqueous methanol. The 2,4-dinitrophenylhydrozone melted at 124°C, and was identified as acetone derivative by mixed m.p.

Oxidation of occidol with alkaline potassium permanganate Occidol (1 g) was oxidized with potassium permanganate (10 g) in a hot alkaline solution containing sodium hydroxide (1 g) for 7 hr. At the completion of oxidation, a filtrate from which manganese dioxide was removed was acidified with dilute hydrochloric acid and evaporated to dryness. The solid residue was extracted with ether in a Soxhlet apparatus until all the organic material was removed. The ethereal extract was evaporated to dryness giving a brownish powder (500 mg), which was purified by chromatography on a column (8 mm × 115 mm) of silicic acid (12 g) (Mallinckrodt) treated with N/2 sulfuric acid (7.5 ml). Elution with *n*-butanol-chloroform (35 : 65) gave white crystals, m.p. 234–236°C, which were esterified with diazomethane in dioxane. The ester thus obtained melted at 129–130°C (lit., 127–130°C). *Anal.* Found: C, 54.94; H. 5.29. Calcd. for $C_{14}H_{14}O_8$: C, 54.19; H. 4.55%.

The experiment on dehydrogenation was carried out by Mr. M. Matsuda, to whom we are indebted. Thanks are also due to the Faculty of Agriculture, University of Tokyo, for infrared analysis and microanalyses. This experiment has been supported in part by grant from the Ministry of Education.

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Action of Lysozyme and Some Microbial Enzymes on Acid-Resistant Organisms

(Studies on Bacteriolytic Substances produced by Microorganisms, Part 5)

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Received August 6, 1958

Cells of an avian tubercle bacillus, when developed into weak acid-fastness were found to be easily susceptible to egg-white lysozyme but not sensitive to bacterial lysozyme. The cells of normal acid-fastness, on the contrary, were resistant to the egg-lysozyme and lysed only by successive application of egg-lysozyme and fungal enzymes. Heat treatment at about 60° changed the acid-fast cells to cells quite sensitive to lysozyme.

INTRODUCTION

The action of lysozyme has long been employed as a useful means in cytochemical studies of many species of bacteria[1),2)], but so far few reports have been published on the study of tubercle bacilli and lysozyme[3)]. Lysis of bacterial cell by lysozyme is attributed to the dissolution of a polysaccharide which plays a part in the maintenance of the cell-wall structure[4)]. Accordingly, it is doubtful whether complete lysis can be expected if this lysozyme sensitive polysaccharide in the cell wall were combined with other cell wall constituents into such a complex in which the susceptible polysaccharide could be protected from the direct attack of lysozyme. Concerning this, the effects of physical or chemical pretreatment of the cells and that of a combined-attack by lysozyme and other enzymes on the lysis were thought to be of special interest[5),6),7)]. Rencently, it has been observed that a lysozyme sensitive polysaccharide really exists in several species of lysozyme-resistant bacteria[6),8)]. Also, the application of enzymes other than lysozyme such as ribonuclease, lipase and phospholipase etc. has become available from studies concerning the composition of cell-wall[9)]. In addition to the enzymatic action, the lysozyme has been known from its basicity to cause an osmotical change to bacterial cells by a certain basic antibacterial polypeptide such as tyrocidin[10)]. Formerly, a killing action of lysozyme for tubercle bacilli had been proved[3),11)], but the existence of lysozyme-substrate polysaccharide has not yet been recognized hitherto. On the other hand, the physiological significance of fat and wax contained in acid-fast cells has attracted attention of some workers and, a lysis of the cells by lipolytic enzyme such as wax-decomposing enzyme "cerase" has been reported[12)].

However, a further detailed investigation would be necessary for the structural features

1) K. Meyer, J. W. Palmer, R. Thompson and D. Khorazo, *J. Biol. Chem.*, **113**, 479 (1936).
2) M. R. J. Salton, *Bacterial. Revs.*, **21**, 82 (1957).
3) Q. N. Myrvik, R. S. Weiser and H. D. Agar, *Am. Rev. Tuberc.*, **67**, 217 (1953).
4) M. R. J. Salton, 3 eme congres international de Biochimie, 27 (1955).
5) G. H. Warren, J. Gray and P. Bartell, *J. Bacteriol.*, **70**, 614 (1955).
6) M. E. Becker and S. E. Hartsell, *Arch. Biochem. Biophys.*, **53**, 402 (1954), **55**, 257 (1955).
7) R. Repaske, *Biochim. Biophys. Acta*, **22**, 189 (1956).
8) R. G. Peterson and S. E. Hartsell, *J. Inf. Diseases*, **96**, 75 (1955).
9) H. W. Dorglas and F. Parker, *Biochem. J.*, **68**, 94 (1958).
10) A. K. Raymond, M. J. Kingkade, S. K. Kern, and O. K. Behrens, *J. Bacteriol.*, **61**, 171 (1951).
11) Q. N. Myrvid, R. S. Weister and M. C. Kelly, *Am. Rev. Tuberc.*, **68**, 584 (1955).
12) M. K. Muftic, *C. A.*, **46**, 7167 (1952), *Enzymologia*, **17**, 222 (1955)

of carbohydrate components of the cell-wall of acid-fast bacilli similary as in the case of many other bacterial cells. In this paper, some aspects of the cell-wall structure of an avian tubercle bacillus are demonstrated, using lysozyme and some microbial enzymes as analytical tools.

EXPERIMENTAL

Methods and Materials.

Cultures and preparation of suspension of washed bacteria; Some relations of acid-fastness with cultural conditions in the same strain of an avian tubercle bacillus are presented in Table I. Bacterial cells of both the acid-fast type and non acid-fast type were employed in the following experiments. Cellswere harvested and resuspended in a phosphate buffer solution after washing and grinding three times with distilled water. The turbidity of the cell-suspension was adjusted to 10% transmission in measuring optical density at 600 mμ with an electric spectrophotometer.

TABLE I. ACID-FASTNESS AND CULTURAL CONDITIONS

Medium	Glucose 2% Polypepton 0.5%	Tween 80 0.5% NH_4Cl 0.1% Salts*
Temperature	37°	37°
Growth-Time	12 hr	48 hr
Shape	rod	spherical
Acid-fastness	−, ±	+

Shaking method was adopted

Salts*: Na_2HPO_4 0.65, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.02% (pH 7.0)

Enzyme preparations: Egg-white lysozyme was crystallized by the method of Alderton[13]. Bacterial lysozyme was obtained from the culture of Bacillus subtilis and purified by the method using ion-exchange resin, previously reported[14]. Enzymes of *Sclerotinia* fungus were extracted from the bran culture with a ten-fold volume of water and the extract was concentrated five times its volume and dialysed. Although the bran culture of *Sclerotinia* fungus possessed a lipase the aqueous extract had no lipase activity[15]. The crystalline trypsin used here, was of an Armers product and commercial pancreatin was used as the extract with water.

13) G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).
14) Y. Satomura, S. Okada and J. Fukumoto, *J. Agr. Chem. Soc. Japan.*, **31**, 801 (1957).
15) Y. Satomura and S. Oi, *J. Agr. Chem. Soc. Japan.*, **31**, 202 (1957).

Measurement of lysis: A suspension of washed bacterial substrate was mixed with the various enzyme solutions and, after adjusting the pH with M/10 phosphate buffer, incubated at 37°. Lysis was followed by the turbidimetric method with the use of a spectrophotometer, or by weighing the dry weight of cells remaining after centrifuging.

Analytical methds: The increase in viscosity was measured by Ostwald's viscosimeter and the degree of liberation of nucleic acid (PNA) was determined by the 'Orcin-HCl' reaction. The release and dissolution of cell-wall mucopolysaccharide was estimated by measuring the turbidity which appeared by the addition of acidified horse serum[16].

Action of Lysozyme

From the experiments shown in Fig. 1. it is distinct that the non acid-fast cells are easily lysed by the action of lysozyme alone whereas, the acid-fast cells are not lysed even by the combined action of trypsin and

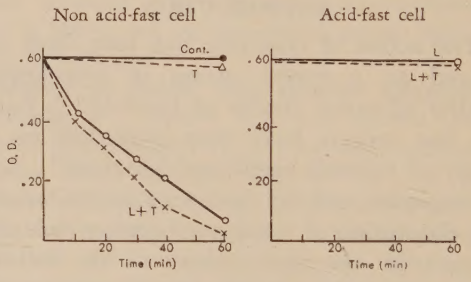


FIG. 1. Susceptibility of Acid-Fast and Non Acid-Fast Cells to Lysozyme (Incubation at 37°)

—○— L, Lysozyme 25 μg/ml
--×-- L+T, Lysozyme 25 μg/ml Trypsin 250 μg/ml
--△-- T, Trypsin 250 μg/ml
—●— Cont. No addition

TABLE II. ADSORPTION OF LYSOZYME TO CELLS

Cell Types	Lysozyme Activity: Initial no addition of cells	Remaining after addition of cells	Rate of Adsorption %
Non Acid-Fast	400	4	99
Acid-Fast	400	300	25
(Lysozyme Non-Sensitive Bacteria *E. coli*)	(400)	(356)	(11)

Treatment

Cell suspension	(10% transmission of optical density at 600 mμ)	10 ml.
Buffer solution		9 ml.
Lysozyme		1 ml.

} at 5° for 5 min.

16) K. Meyer, *Physiol. Revs.*, **27**, 335 (1947).

lysozyme. In Table II, the difference in the degree of lysozyme adsorption of both types of cells is given. Thus, it can be supposed that the polysaccharide-substrate of lysozyme surely exists in cells of the non acid-fast type, as observed in the other lysozyme-sensitive bacterial cells which readily adsorb lysozyme.

Joint Action of Lysozyme with other Enzymes

As seen in Table III, egg-white lysozyme dissolves only 22~23% of the weight of the acid-fast cells even in a prolonged period of 18~20 hr. at pH 6.0, while the enzyme solution of *Sclerotinia* digests cells up to about 50% in the same conditions. Bacterial lysozyme possesses only scanty activity. However, a certain combined-use of these agents gave a particulary accelerated lysis action. When the *Sclerotinia* extract was combined with egg-white lysozyme in the above condition, it digested the cells up to ca. 70%, which is equal to the sum of lysis power of both agents. When however, the cells were pretreated with lysozyme for about 2 hrs. at pH 6.0 and centrifuged, resuspended in a buffer of pH 4.0 and then successively treated with *Sclerotinia* extract, lysis extended up to more than 80% within a period of 4 hours. From the results obtained, it was supposed that an inhibitory effect on the action of lysozyme had been caused by an acidic substance contained in the fungus extract, which can combine with lysozyme. In the same procedure, pancreatin pretreatment plus *Sclerotinia* extract attained a ca. 60% lysis, and bacterial lysozyme plus *Sclerotinia* extract ca. 55% lysis. In these experiments *Sclerotinia* extract lost its activity upon heating at 100° for 10 minutes.

It was also demonstrated that the *Sclerotinia* enzyme extract attacked the polysaccharide fraction of the acid-fast cells to produce a small quantity of reducing sugar, decreasing the viscosity of the solution. As main sugars of the polysaccharide constitutents glucosamine, glucose, mannose and rhamnose were detected by a paper-chromatographic test after acid hydrolysis.

TABLE III. SINGLE OR JOINT ACTION OF LYSOZYME AND OTHER ENZYMES ON THE CELLS OF ACID-FAST TYPE

Cell Suspension +			Rate of Lysis
	S	18~20 hr (pH 6.0) 37°	46.0%
		(pH 4.0)	51.8
+	L	18~20 hr (pH 6.0) 37°	22.5
+	Panc.	〃	19.5
+	Try.	〃	2.0
+	Bact. L	〃	9.5
+	(S+L)	〃	70.6
+	L	2 hr (pH 6.0) S 4 hr (pH 4.0)	83.2
+	Panc.	〃 S 〃	59.7
+	Bact. L	〃 S 〃	54.8

(in Final concentration)

S: Sclerotinia fungus-enzyme, 10 times water extracts of bran culture
L: Egg-white lysozyme, 30 μg/ml
Panc.: Pancreatin, 1% aqueous extract
Try: Trypsin, 100 μg/ml
Bact. L: Bacterial lysozyme ca. 50 μg/ml

Rate of Lysis = Cell-remains $\left(\frac{\text{Control}-\text{Test}}{\text{control}}\right)\times 100$

Susceptibility of Heated Cells to Lysozyme

As described above, the sensitivity of acid-fast cells to lysozyme was very weak in an intact state, but sensitivity was found to be increased by heating the cells at about 60° for 10 min. in distilled water, thus bringing about the Nakamura effect[17]. As shown in Fig. 2, the heated cell-suspension rather increases its turbidity in the earlier stage of incubation with lysozyme, as in the case with heat-killed cells of other lysozyme sensitive or non sensitive bacteria[2], but prompt lysis occurs upon the addition of NaOH changing the pH from 6 to 12, concurrently increasing viscosity. However, the Nakamura effect did not appear with the cells heated at temperatures lower than 50° or higher than 70°. Also, the effect was not observed with the cells previously treated with a dilute HCl or NaOH solution. The degree of clearing, viscosity and the amount of nucleic acid released from the cells, increased in parallel

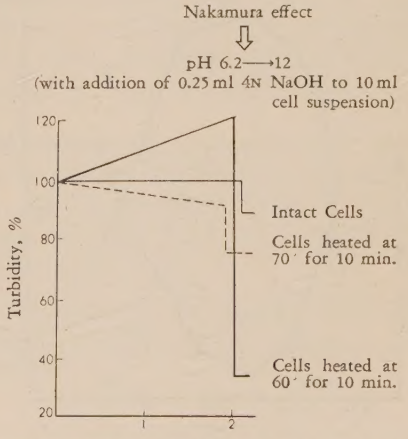
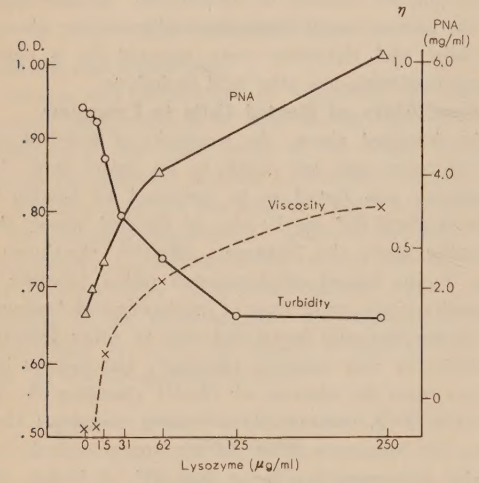


FIG. 2. Increase in Susceptibility of Cells to Lysozyme by Heat-Treatment, bringing about Nakamura Effect

17) O. Nakamura Z. *Immunitatsforsch.*, **38**, 425 (1923).

with the concentration of lysozyme added to the cell suspension, as presented in Fig. 3. As shown in Fig. 4, from the activity of clearing and releasing-PNA, the optimum pH of lysozyme action is about 6.0, while from the activity of increase in viscosity, the optimum pH seems to be in the vicinity of 7.0. In an incubation with lysozyme at 40° for 2 hr., the supernatant of the cell-suspension was found to give rise to turbidity



(incubation with cells for 2 hr)

FIG. 3. Relation of Lysozyme—Concentration with Nakamura Effect on Cells heated at 60° for 10 min.

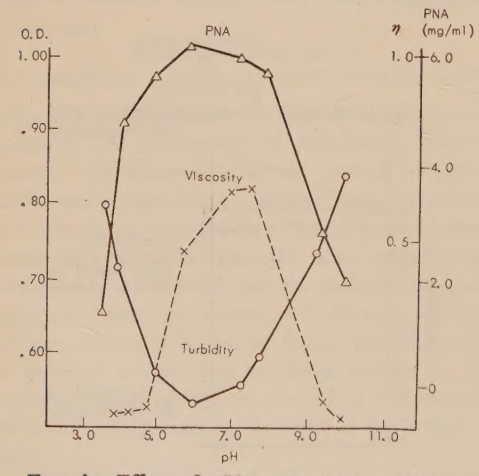


FIG. 4. Effect of pH on Sensitivity of Heated Cells (60°, 10 min.) to Lysozyme, bringing Nakamura Effect

(during incubation at a conc. of 125 μg lysozyme/ml, for 2 hr)

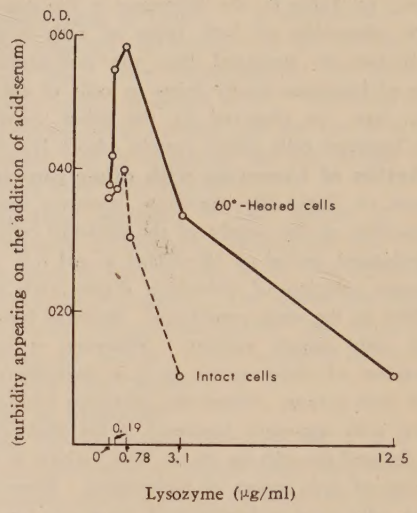


FIG. 5. Release and Dissolution of Cell-Wall Mucopolysaccharide during Incubation with Lysozyme

upon the addition of an acidified horse serum. Hence, a certain kind of mucopolysaccharide was considered to be dissolved-out from the cell-walls during incubation. In Fig. 5 the turbidity appearing in such treatment with various concentrations of lysozyme is shown. It is noticeable, that in the curve there seems to exist optimal concentration to give maximal turbidity and an excess of lysozyme decrease, in proportion to remarkably the concentration, the turbidity. This phenomenon is most probably due to hydrolysis of mucopolysaccharide by an excess of lysozyme into the non-precipitable fraction with acid serum. With intact cells, the dissociation of mucopolysaccharide was found to be far weaker than that of heated cells. Also, the mucopolysaccharide revealed to contain a large amount of glucosamine as its main sugar component, by a paperchromatography following acid hydrolysis.

DISCUSSION

The significance of effects of heat-treatment on susceptibility of acid-fast cells to lysozyme was recognized in the heat-treatment at about 60°. W. B. Redmond and his coworker[18] have reported on the autolysis of a tubercle bacillus grown in a nitrogen-deficient medium where autolytic enzymes were either unaffected or

18) W. B. Redmond and B. U. Bowman, *J. Bacteriol.*, **69**, 293 (1955).

little affected by heating at 56° for 30 min. In some gram-negative bacteria, it has been demonstrated that disorganization of the cell-wall brought about by physico-chemical[5),6),7)] or a certain antibiotic treatment, results in the acceptance of lysozyme action[19)]. Howere, the polysaccharide substrate of lysozyme in the acid-fast cells present in the complex with other cellular constituents may not be considered to accept the action of lysozyme directly, after undergoing any change in the permeability-barriers by heat-treatment, as has been mentioned previously by M. E. Becker and S. E. Hartsell[6)]. It seems reasonable to assume that some autolytic enzymes which can split the complex structure of cell-surface substances would be activated by the heat-treatment, whereby a structural-site for the acceptance of lysozyme appears.

The difference between the action of egg-white and bacterial lysozyme on acid-fast cells indicates that the former enzyme[20)] has a comparatively broad specificity as a polyglucosaminidase for the bacterial substrates while the latter a somewhat limited specificity. From this fact, the use of the bacterial lysozyme may offer a new tool for a more fundamentary study of the chemical constitution of bacterial cell-wall susceptible to lysozyme.

From the experiments described above, the main factors causing the synergistic effect within lysozyme and other enzymes are not considered to involve any of the following enzymes: protease, nuclease, lipase, phospholipase and esterases. But the factors are thought to be some sort of polysaccharidase commonly found in fungi just like those of *Sclerotinia* fungus, such as chitinase, cellulase, lichenase, polyglucosanase[21)] and protopectinase etc. Recently, A. Sohler and his coworkers[22)] reported that the different sensitivities of *Actinomycetales* to lysozyme, could be explained by the difference in carbohydrate composition of the cell-wall, and the lysozyme-resistant strain contained only a small amount of hexosamine contrary to its richness in arabinose and galactose. Lysozyme and other lytic factors should operate alternatively to dissolute the complex structure of the cell-wall, stepwisely removing the obstructive sites.

19) G. H. Warren, J. Gray and J. A. Yurchenco, *J. Bacteriol.*, **74** 788 (1957).
20) L. R. Berger and R. S. Weiser, *Biochim. Biophys. Acta*, **26**, 517 (1957).
21) R. A. Aitken, B. P. Eddy, M. Ingram and C. Weurman, *Biochem. J.*, **64**, 63 (1957).
22) A. Sohler, A. H. Romano and W. J. Nickerson, *J. Bacteriol.*, **75**, 283 (1958).

SUMMARY

(1) Non acid-fast cells of an avian tubercle bacillus were easily lysed by lysozyme, whereas acid-fast cells could be digested by a successive attack of lysozyme followed by *Sclerotinia*-enzyme. Bacterical lysozyme, trypsin and pancreatin, however, had no synergistic action with lysozyme. The synergistic factor contained in the *Sclerotinia*-enzyme extract was assumed to be a certain polysaccharidase.

2) The release of a mucopolysaccharide from the cell-wall during incubation with lysozyme increased by previously heating the cells at about 60° for 10 min. The effect of heat-treatment upon the sensitivity of cells to lysozyme, which was accompanied by a remarkable Nakamura effect, was concluded to be due to the activation of some autolytic enzymes.

The authors wish to express their thanks to Dr. I. Yamasaki, for reading this manuscript. They are also indebted to Mr. Y. Nishikawa and Mr. M. Ono, for their assistance in part of these experiments.

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Biochemical Investigations on the Lipase Formation of *Sclerotinia* Fungus and on the Specificity of Lipase Action of Some Fungal Origins

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Received August 18, 1958

The lipase forming activity of *Sclerotinia Libertiana* increasing with the development of the conversion system of fat into carbohydrate in the cells, was convinced to have a relation with the ability of oxidative fermentation of fungus strains.

Although complete hydrolysis of fat was not attained by the single action of a fungal lipase such as of *Sclerotinia* and *Rhizopus*, it was found to be completed with an appropriate combination of both enzymes. The difference in specificities of the enzymes towards a substrate other than glyceride and the activities in organic solvent such as ether or petroether are demonstrated in this paper.

INTRODUCTION

Although some species of the moulds isolated from the fatty materials produced lipase substantially[1], and that in certain strains lipase-forming activity could be increased by adding fat to the medium as a nutrient[2], in case of *Mucorales*, a high lipase-forming activity has been generally recognized through the genus, usually showing no effect of fat[3] added. *Sclerotinia Libertiana* Fcl. was also found to produce lipase abundantly without addition of fat and, in this case, lipase, was formed by washed mycelium during shaking in a plain buffer solution, similar to that in the shaking culture[4].

Among fat producing microorganisms, some are powerful to form lipase[5]. However, no distinct relationship between the function of fat-production and the formation of lipase has yet been proved. With respect to the physiology of lipase-producing fungi, it is noticeable that their aerobic fermentability was in general found to be comparatively strong.

The conversion of fat into carbohydrate which has been observed in germinating seeds[6], such as castor beans, seems to present an important suggestion on the mechanism of metabolic changes of reserved fatty materials in lipase-forming microorganisms. In the previous report[4], the authors have shown that the lipase formation by *Sclerotinia* proceeds in proportional to the consumption of reserved fat in the cells. In this paper, it is concluded that lipase formation is caused by the metabolic conversion of fat into carbohydrate.

Many points having relation to the mechanism of lipase action still remain obscure, for example, it is not decided whether the 2-monoglyceride, remaining in the course of hydrolysis of triglyceride by pancreatic lipase can be further hydrolysed directly, or subsequent to firstly being isomerized to the 1-form[7]. On

* Osaka Bacteriological Research Institute Ltd.

1) C. V. Ramakrishnan and B. N. Banerjee, *Enzymologia*, **XV**, 33 (1952).

2) J. J. Goodman, *Science*, **112**, 76 (1950).

3) C. Matsumura, Meeting of Agr. Chem. Soc. Jap. (1956).

4) Y. Satomura and S. Oi, *J. Agr. Chem. Soc. Jap.*, **33**, 202 (1952).
Y. Satomura, S. Oi and A. Sawada, This Bulletin, **22**, 194 (1958).

5) I. I. Peters and F. E. Nelson, *J. Bacteriol.*, **55**, 581 (1948).

6) H. L. Kornberg and H. Beevers, *Biochim., Biophys. Acta*, **26**, 531 (1957).

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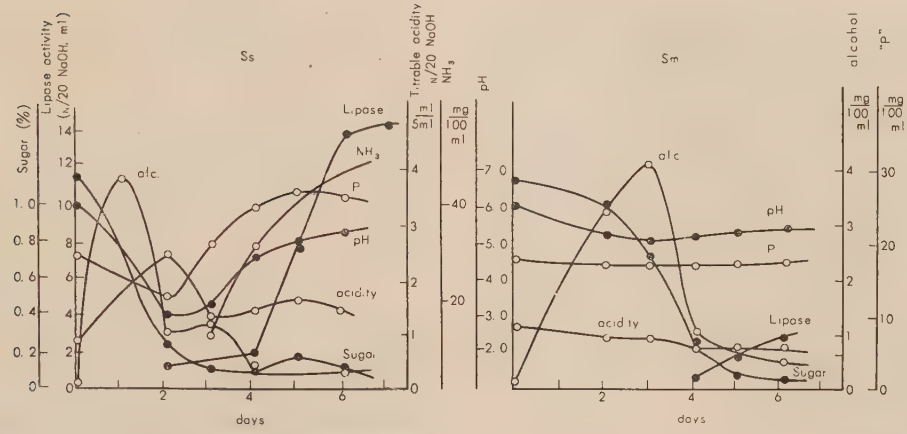

FIG. 1. Relation of Sugar Consumption, Alcohol Formation, pH change, Titrable Acidity, and Inorg. Phosphate or NH_3 Liberation with Lipase Formation

(shaking culture in a medium of 5% decoction of bran without addition of $CaCO_3$)

the other hand, it has been reported that oat lipase attacks only the 2-ester bond of triglyceride and is unable to split either di- or monoglyceride[8]. Studies of this kind regarding fungal lipase, are very few[9]. Thus, the mode of hydrolysis of fat by some fungal lipase was investigated and the specificities for some substrate other than glyceride and the activities in the organic solvent such as ether or petroether were also examined.

METHODS

Strains, their culture and preparation of washed mycelium:

Sclerotinia Libertiana Fcl. Bran culture and shaking liquid culture were adopted, of which the latter medium consisted of 5% decoction of wheat bran and 0.3% $CaCO_3$. To study the physiology of lipase formation, the original strain Ss, of high lipase-forming activity, and its mutant Sm, of weaker activity were used and compared. Washed mycelium was prepared by harvesting cells after about 4 days of shaking culture, centrifuging and washing them three times with 0.6% NaCl.

Rhizopus delemar The organism was cultured in a medium containing 4% dextrin, 1% $(NH_4)_2SO_4$, 5% corn steep liquor and 1.5% $CaCO_3$ on the shaker.

Mucor mucedo Culture on bran was undertaken.

Enzyme preparations: Since *Sclerotinia* lipase is an endocellular enzyme, the dry mycelial powder of bran culture or the suspension of washed mycelium obtained by shaking culture was employed as the enzyme preparation. *Rhizopus* lipase which is excretive in the medium was used in the state of culture filtrate. *Mucor* lipase was extracted from the bran culture with a tenfold volume of water.

Lipase activity was measured in the following way: Reaction mixture consisting of the enzyme preparation, 1 ml of olive oil, 2 ml McIlvain buffer solution (pH 7.0) and 5–10 ml M/50 $CaCl_2$ solution was shaken at 37° for a desirable time, and the contents were taken out then, after addition of 25–30 ml alcohol the liberated fatty acid was titrated against N/20 NaOH at about 55°. At the same time, two blank tests, one with a heat inactivated enzyme, the other without substrate oil were performed. The titration value subtracted by both two blank tests represents lipase activity.

Cell constituent;

Carbohydrate Total reducing sugar was measured after hydrolysing the mycelium with 1N HCl for 2 hr.

Fat After hydrolysis of mycelium under the same condition as above, total ether-extractable matter of the digest was measured.

EXPERIMENTAL AND RESULTS

Comparative Physiology of *Sclerotinia* Fungus strains.

As shown in Fig. 1, a small amount of alcohol was formed at an early-stage of culture in both strains of

8) F. G. Peers, *Nature*, **171**, 981 (1935).
9) J. V. Fiore and F. F. Nord, *Arch. Biochem.*, **26**, 382 (1950). K. Lakshminarayanan, *Enzymologia*, **19**, 59 (1958).

Ss and Sm, but soon disappeared and, in the subsequent stage titration acidity was found to increase in Ss, but not in Sm. The organic acids detected in the culture of Ss by paper chromatography were mainly citric and oxalic acid, and small amounts of fumaric, succinic and malonic acid were also found. Thereafter, a decrease in acidity occurred followed by liberation of inorganic phosphate. The lowering of pH in Ss in the early period seemed to depend on the formation of organic acids and on the liberation of inorganic phosphates. The pH of the culture then gradually rose with the appearance of NH_3. At this time lipase formation began to occur abruptly. In Sm, in which the pH change was quite insensitive only a very weak lipase formation took place.

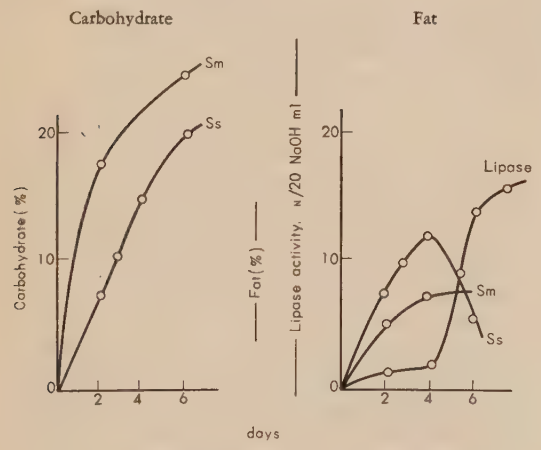

FIG. 2. Change in Percent of Carbohydrate and Fat in the Cell

As shown in Fig. 2, the carbohydrate content in both Ss and Sm increased progressibly in the whole course of culture and the accumulation of carbohydrate was greater in Sm than in Ss. However, the fat content of Ss which increased far more than that of Sm in parallel with the change of carbohydrate at the early-stage, suddenly turned to show a decrease with the occurrence of lipase formation. Whereas, in Sm the fat content did not change, maintaining a value which was almost constant in the later-stage corresponding to a poor lipase formation.

In table I, the value of respiratory quotient (RQ) and the oxygen uptake of both strains are given. From this result it may be recognized that Ss is more oxidative and smaller in RQ than Sm at an early-stage, but later the RQ values gradually increased until a value greater than 1.0 is attained. In connection with this,

TABLE I. RQ AND O_2 UPTAKE OF Ss AND Sm ON AGAR CULTURE IN WARBURG FLASK

		days 1	3	4	5	6	7	8
RQ	Ss	0.70	0.63	1.22	1.38	1.33	1.42	1.31
	Sm	1.23	1.19	1.31	1.34	1.37		
O_2 uptake μl/hr	Ss	5.5	158.0	246.0	45.0	78.5	88.0	76.5
	Sm	3.6	153.0	93.5	102.0	90.0		
Q_{O_2}	Ss			5.2				
	Sm		3.4					

it was assumed that the evolution of CO_2 due to the degradation of the accumulated oxalic acid in the culture would increase the apparent RQ value as shown in the following experiments.

Metabolic Change of *Sclerotinia* Washed Mycelium during Lipase Formation

As shown in Fig. 3, the decrease in fat content with the development of lipase formation is very distinct in the shaking experiment of Ss in a plain buffer solution. In this case, a small amount of alcohol is also formed, but later on oxalic acid is shown to be formed by paperchromatography.

As observed in Fig. 4, cyanide and bile salt had no inhibiting effect, in spite of the remarkable inhibition on oxygen consumption of washed mycelium, but rather an increasing effect on the enzyme formation. Especially, the effect of the latter reagent was distinct. The ad-

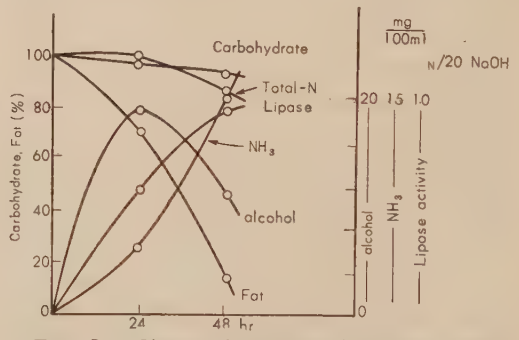

FIG. 3. Change in Reserved Cell-Materials, Alcohol Formation and Lipase Formation in Washed Mycelium during Shaking in Plain Buffer Solution

dition of dialysed enzymic solution extracted from the bran culture of Ss itself, gave the most promoting effect on lipase activity. This effect of the dialysed enzymic solution remained almost intact even after heating at 100°, 10 min.

The RQ value of washed mycelium in the shaking experiment increased gradually with the time of shaking,

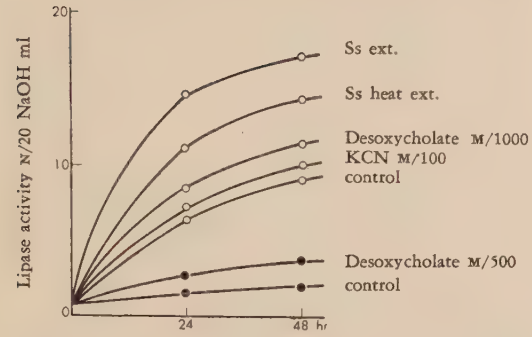

FIG. 4. Effect of Cyanide, Desoxycholate and Ss Extract on Lipase Formation by Washed Mycelium.
Ss extract was added as 0.1% in final concentration of dry mycelial weight.

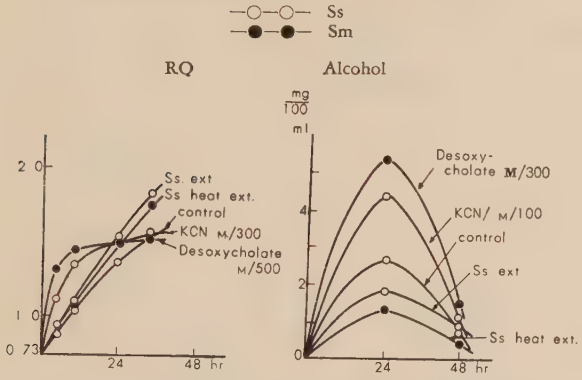

FIG. 5. Change in RQ and Alcohol Formation with Addition of Cyanide, Desoxycholate and Ss Extract to Washed Mycelium.

as shown in Fig. 5. Addition of cyanide and desoxycholate immediately elevated the RQ value, accompanying an increase in alcohol formation. But the Ss enzymic solution, in spite of its non-influence of alcohol formation and oxygen consumption, increased the RQ value most remarkably.

Then, it was found that the washed mycelium can decompose oxalic acid evolving CO_2, as prerented in Table II. Also, formic acid was detected by $HgCl_2$ in the distillate of the reaction mixture of oxalic acid

TABLE II. DEGRADATION OF OXALIC ACID BY *Sclerotinia*

	O_2 uptake	CO_2 evolved	RQ
control	20.3	14.3	0.70
oxal. M/400	25.0	22.5	0.90

in warburg flask at pH 4.5 by washed mycelium harvested at 4 days age of shaking culture

with the mycelium. Therefore, the increase in RQ value during shaking was in part attributable to the degradation of oxalic acid accumulated in the reaction mixture.

Modes of Fat Hydrolysis and its Specificities

Hydrolysing curves of olive oil by lipase of *Sclerotinia*, *Rhizopus* and pancreatic origin are shown in Fig. 6. The curve representing *Rhizopus* lipase well resembled that of pancreatic lipase, the reaction proceeded rapidly at

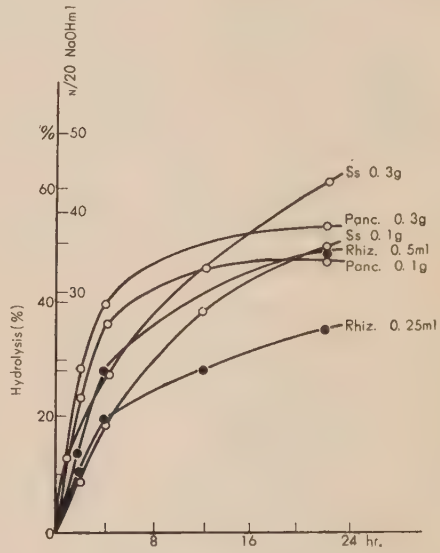

FIG. 6. Hydrolysis Curve of Olive oil by Lipase of *Sclerotinia*, *Rhizopus* and Pancreas Origin.

first, but towards nearly 8 hours the majority of the peak-zone of hydrolysis was 60%, at a high concentration of the enzymes. On the contrary, the activity of *Sclerotinia* lipase developed a steady progress, though it was a little slower than that of *Rhizopus* or pancreatic lipase in the earlier period, finally reaching a higher extent of hydrolysis exceeding 60%.

The progress curves obtained by using combined exzymes are shown in Fig. 7. When *Sclerotinia* lipase was combined with either *Rhizopus* or pancreatic lipase, the rate of hydrolysis readily proceeded to an extent of more than 60% and, in 48 hr it exceeded 95%. Since each enzyme itself, even at its double concentration under the same condition could not attain 60% hydrolysis, the observed effect in the joint action may be considered as an additive effect exerted together by each single action of the enzymes. However, the combination of *Rhizopus* lipase with *Mucor* or pancreatic

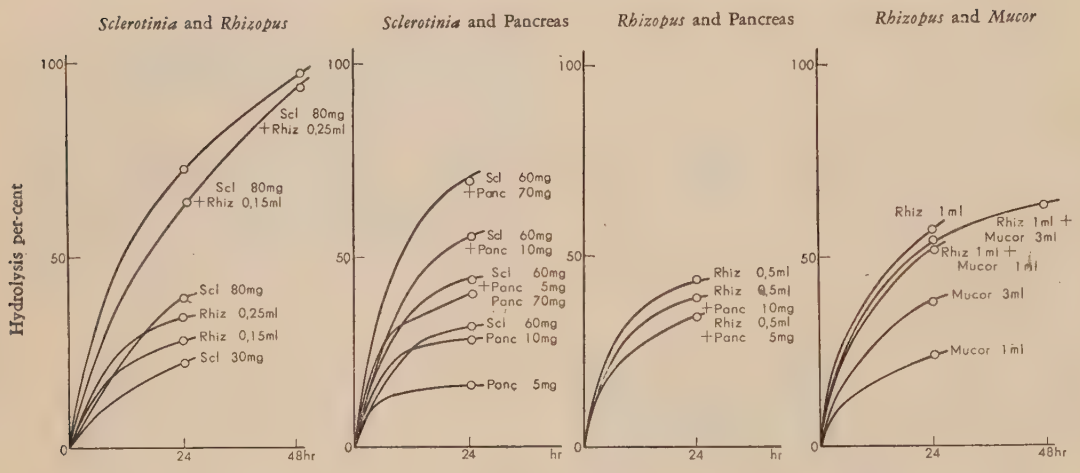

FIG. 7. Progress Curve of Hydrolysing Olive Oil by Enzymes mixture.

TABLE III. ACTIVITIES IN ORGANIC SOLVENTS

Subst. \ Enzymes / Solu.		Ss (bran culture) 0.2 g	*Rhizopus* 0.5 ml	*Mucor* 2 ml	Pancreatin 0.1 g	Reaction time (hr.)
Olive oil 1 g	Aqueous	26.7	35.0	16.0	28.0	(22)
	Ethylether	2.15	4.5	0	0.5	(22)
	Petroether	4.12	11.9	0	2.9	(72)
Tristearin 0.5 g	Ethylether	1.5	—	—	0.5	(22)
	Petroether	7.0	6.75	—	1.2	(72)

lipase did not show such additive effect.

In Table III activities of these lipase in organic solvents such as ether or petroether, as measured in the case with lecithinase A[10], are presented. It is noteworthy that *Sclerotinia* and *Rhizopus* lipase have considerable activity even in such organic solvents, especially the activity of the former being distinct on tristearin.

Specifities for the non-glyceride fatty acid ester such as Tween and Span are given in Table IV. The activity of *Sclerotinia* lipase was found to be most powerful and specific, and thereat the longer the chain length of fatty acid the more easily it was hydrolysed. However *Rhizopus* or *Mucor* lipase had a rather low affinity towards a high fatty acid eater as observed in Tween 80 or Span.

TABLE IV. SPECIFICITIES FOR TWEEN AND SPAN

Enzyme / Subst. (1 ml.)	Ss (bran culture) 0.2 g	Pan-creatin 0.1 g	*Rhizopus* 0.5 ml	*Mucor* 0.2 g
Tween 20	11.8	1.9	4.7	10.0
Tween 40	21.7	13.6	10.4	13.5
Tween 80	21.4	7.7	9.8	8.3
Span 80	34.4	15.8	13.1	8.4
Olive oil	26.7	28.0	35.5	16.6

10) D. J. Hanahan, *J. Biol. Chem.*, **195**, 199 (1952).

DISCUSSION

From the high RQ value and the alcohol formation observed at the stage of rapid fat consumption by washed mycelium, it is reasonable to assume that the carbohydrate is directly consumed and serves as an energy source partly via the glycolytic pathway, and that the decrease of fat is due to its conversion into carbohydrate in the cells. Thus, the lipase fromation can be considered to link the conversion of fat into carbohydrate in the cells. The

promoting effect of bile salt on lipase activity may be ascribed to its accelerating effect on glycolysis in the cell, and to the effect of dispersing lipid rendering it more accessible to enzymes in the cell. From the effect of cyanide on both oxygen uptake and lipase activity, it can be deduced that a very limited quantity of respiration would be sufficient to bringforth the lipase formation.

Recently, the significance of the glyoxylate cycle in metabolism of bacteria, fungi and seeds containing fat has been demonstrated[6),11),12)]. Probably, in *Sclerotinia* the reaction system from fat to carbohydrate proceeds via the glyoxylate cycle, and also oxalic acid may be formed via glyoxylic acid. It seems to be interesting that this fungus has a faculty of decomposing oxalic acid as it has been reported with some plant pathogenic fungi[13),14)]. The increasing effect of Ss enzymic solution on the RQ value of washed mycelium should be ascribed to the acceleration of oxalic acid formation thereby developing the glyoxylate cycle, consequently leading to the promotion of conversion of fat into carbohydrate, further proceeding to the enhancement of lipase formation. Concering this fact, further investigations on the factors involved in the Ss enzymic solution are necessary. The reason for the mutant strain Sm which shows no activity of oxidative fermentation being so weak in the ability of lipase formation, may be explained by the relationship between the development of conversion system of fat into carbohydrate and the activity of oxidative fermentation.

From the result of comparison of hydrolysing curves of olive oil by fungal enzymes and the fact of a high activity of *Sclerotinia* lipase on the non-glyceride mono fatty acid ester, it is suggested that the remarkable additive effect observed on olive oilve oil of *Sclerotinia* lipase to either *Rhizopus* or pancreatic lipase is probably due to the prompt hydrolytic action of 2-mono fatty acid ester of glyceride by the former. However, such joint effect observable in the combination of two enzyme actions which differ from each other in the specificities for substrates would vary according to the variety of fat and thereat, as seen in the case with the combination of *Rhizopus* lipase and pancreatic or *Mucor* lipase, the occurrence of some interaction which rather mutually inhibits each enzyme activity in the course of combined reaction may be conceivable. The difference in the activities of enzymes in organic solvent seems to have no relation with the modes of the hydrolysing action of fat, but is probably related with the physico-chemical properties of enzyme-proteins.

11) H. L. Kornberg and J. F. Collins, *Bioch. J.*, **68**, proc. 3 p. (1958).
12) H. L. Kornberg, *Bioch. J.*, **68**, 535 (1958).
13) H. Shimazono, *J. Biochem.*, **42**, 321 (1955).
14) Y. Nagata and K. Hayashi, *J. Agr. Chem. Soc. Jap.* **31**, 575 (1957).

SUMMARY

1. In biochemical investigations on the lipase formation of *Sclerotinia* fungus, the following facts were observed. i, The original strain Ss had an activity of oxidative fermentation while its mutant Sm had not such activity; the activity of the former seemed responsible for its high activity of lipase formation in a later-period of culture. ii, During the lipase formation by washed mycelium of Ss at the expense of reserved fat maintaining its carbohydrate content at a constant level in the cell, the production of alcohol and oxalic acid were recognized and the RQ value exceeded 1.0. iii, Though bile salt and cyanide were inhibitory for the endogeneous respiration they were stimulatory for the fermentation of cellular carbohydrate and the lipase activity of washed mycelium was increased to some extent. iv, The extract of bran culture of Ss itself, remarkably promoted the lipase activity of washed mycelium along with increase of RQ value and had no influence on oxygen consumption. v, The washed mycelium of Ss harvested at a later period of culture was found to decompose oxalic acid into CO_2 and formic acid.

2. The lipase type of the following fungi

was proved to be dividable into two groups; One is that of *Sclerotinia* which can react additively on olive oil with either *Rhizopus* or pancreatic lipase which also has a high activity on substrates other than glyceride such as Tween ann Span, and the other is that of *Rhizopus* which can not react additively with pancreatic lipase.

3. The fungal lipase was shown to have considerable activity in organic solvents such as ether or petroether.

The authors wish to express their thanks to Dr. I. Yamasaki for his careful revision, and to Mr. N. Yoshizu, Director of Osaka Bacteriological Research Institute for his help. They are also indebted to Mr. H. Ishii for his assistance in part of the experiments.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 156~159, 1959]

Fractionation of Maltase and Saccharogenic Amylase in Mold, and Crystallization of Maltase

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Two enzymes which are concerned with maltose hydrolysis in molds, saccharogenic amylase and maltase, were fractionated by adsorption with calcium triphosphate and elution with potassium-diphosphate and diammoniumphosphate solutions. Maltase was crystallized from diammonium-phosphate eluate by a method involving fractionation with ethanol and acetone, and the removal of salted-out inpurities. It was found that the crystalline maltase hydrolyzes maltose and phenol-α-glucoside but methyl-α-glucoside is not appreciably hydrolyzed, while saccharogenic amylase is inactive toward the latter two substrates. Optimal pH of crystalline maltase is 3.4 and opt. temperature 50°C.

It has been reported by many workers that mold saccharogenic amylase hydrolyzes maltose in addition to starch[1~5]. Thus, in studies of saccharogenic enzymes systems in molds it is a significant problem whether the maltose hydrolysis is attributed to this enzyme alone or to the presence of maltase-like substances in yeast and higher plants.

The authors have succeeded in the fractionation of saccharogenic amylase and maltase activities by treating "koji" extract with calcium-triphosphate and subsequent elution with potassiumdiphosphate and diammoniumphosphate solutions, and crystalline maltase was isolated by further-purification from the latter eluate. (Crystalline mold maltase has most recently been reported by Tujisaka and Fukumoto[6]).

This enzyme has no activity of starch hydrolysis and hydrolyzes maltose and phenol-α-glucoside but not appreciably methyl-α-glucoside.

1) H. Okazaki, *J. Agr. Chem. Soc. Japan*, **28**, 48, 51 (1954).
2) R. W. Kerr et al., *J. Agr. Chem. Soc.*, **73** 3916 (1951).
3) L. L. Phillips et al., *J. Am. Chem., Soc.*, **73**, 3559, 3563 (1951).
4) J. Fukumoto et al., *Symp. on Enz. Chem. (Japan)* **9**, 94, (1954).
5) S. Hayashida, Meeting of Agr. Chem. Soc. in Japan (1958).
6) Y. Tujisaka et al., *Symp. on Enz. Chem. (Japan)* **10**, 84 (1958).

The present paper deals with the fractionation of maltose-attacking enzymes and crystallization of maltase, substrate specificity, and [illegible] of the crystalline maltase.

EXPERIMENTAL

Methods and materials.

Aspergillus oryzae "koji" was used as the source material. Calcium-triphosphate-gel was prepared according to the method of Keilin & Hartree[7].

Maltase activity was measured by the increase of reducing power, adopting a modification of the [illegible] method devised by Kobayashi et al.[8]

Preparation of α-amylase-free enzyme solution from "koji", and fractionation of saccharogenic amylase and maltase.

Enzymes were extracted with 50% ethanol. The intention of the procedure was the removal of α-amylase. That is, α-amylase was adsorbed by residual starch of "Koji" in a fifty percent alcoholic solution, while maltase and saccharogenic amylase were not adsorbed[9].

When α-amylase was contaminated in the 50% ethanol extract, [illegible] with residual starch of Koji (The extracted residue was washed thoroughly with 1% sodium chloride) was repeated.

As is shown in Table I these two enzymes, saccharogenic amylase and maltase, are completely [illegible]

TABLE I. FRACTIONATION OF MOLD SACCHAROGENIC AMYLASE AND MALTASE

Powdered rice "Koji" 3 kg.

↓ [illegible]

50% ethanol extract

↓ added equal volume of ethanol, [illegible]

Precipitate

↓ dissolved in 350 ml. of water, added kaolin and pH adjusted to 6.6~6.8 with N/25 NaOH, centrifuged,

[illegible]

↓ neutralized to pH 6.6~6.8 with N-NaOH, passed through a column of calcium-triphosphate gel, mixed with an equal amount of Celite, washed with water,

Calcium triphosphate-celite column

[illegible] collected each 20 ml. of eluate until maltose-decomposing activity disappeared

↓ ↓

M/15 KH_2PO_4 fraction | M/10 $(NH_4)_2HPO_4$ fraction

(Saccharogenic amylase) | (Maltase)

7) D. Keilin et al., [illegible]
8) T. Kobayashi et al., J. Agr. Chem. Soc. Japan, 28, 171 (1954).
9) Y. Tsujisaka, J. Brew. Soc. Japan, 14, 279 (1956).

by acetic and basic phosphate respectively.

The order of the elution may follow the above-mentioned definition, since diammonium-phosphate eluates [illegible]

No concentration of diammonium-phosphate solution higher than 0.1 M was required to reduce the elution [illegible]

Elution was made by collecting 20 ml.-portions of each eluate and in case further purification and crystallization were planned, parts having weak activities were [illegible]. Table I is an example of the procedure, and [illegible] maltase activities obtained in each stage of purification is shown in Table III.

Further purification and crystallization of maltase from diammoniumphosphate eluates.

The purpose of treatment with Amberite IR 45 was the exchange of phosphate or sulfate for acetate.

The large quantities of impurities were precipitated by salting-out with ammonium sulfate while maltase still remained in the solution. It is not clear whether this behaviour of maltase to salting-out effectiveness is due to the [illegible] condition or its specific property.

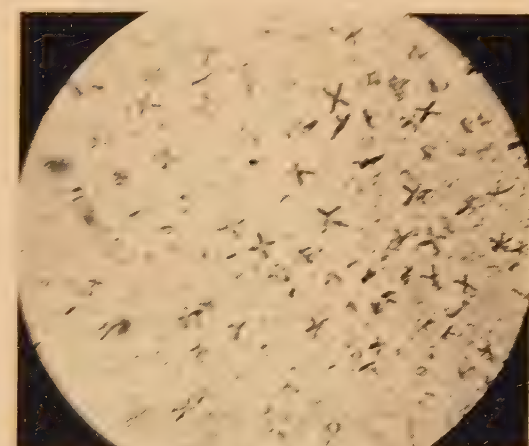

FIG. 1

At the final stage of purification, acetone was used instead of ethanol and, by the use of this solvent crystallization occurred. Crystals of the enzyme appeared from five to fifteen hours in the ice box. For recrystallization, this precipitant was dissolved in a minimum volume of water and acetone was added until the solution became turbid at 0°C. Further addition of acetone was made after turbidity disappeared and this procedure was repeated until the concentration of acetone increased about 60%.

TABLE II. CRYSTALLIZATION OF MOLD MALTASE FROM DIAMMONIUMPHOSPHATE FRACTION.

Diammoniumphosphate Eluate

↓ Amberite IR 45 (pre-treated with sodium acetate) treatment, concentrated under diminished pressure, added alcohol, the 50~70% alcohol precipitate dissolved in 50 ml. of 0.5 M acetate (pH 5.0) and alcohol (70%) treatment repeated.

Precipitate

↓ dissolved into a minimum quantity of 0.5 M-acetate, added a 4-fold volume of sat. $(NH_4)_2SO_4$ sol., precipitate discarded.

Filtrate

↓ dialyzed against running water (3~4 days), treated with Amberite IR 45, precipitated with alcohol (75%).

Precipitate

↓ dissolved in 25 ml. of 0.5 M acetate (pH 5.0), discarded insoluble substances by centrifugation, added an equal volume of acetone, centrifuged.

Supernatant

↓ further addition of acetone up to the concentration of 67%, stood an ice box.

Crystalline maltase.

TABLE III. YIELDS OF MALTASE ACTIVITY IN EACH STAGE OF PURIFICATION.

		recovery against 50% ethanol extract (%).
1.	50% ethanol extract	100
2.	75% ethanol ppt.	95~97
3.	M/10 $(NH_4)_2HPO_4$ eluate (only parts having strong activity collected)	50~60
4.	70% ethanol ppt.	48~55
5.	dialyzed solution	50~55
6.	50% acetone ppt.	20~36
7.	1st crystallization	8~20

Complete removal of the amorphous substances was however difficult after three recrystallizations.

Substrate-specificity, optimal pH and optimal temperature of crystalline maltase, and comparison with purified saccharogenic amylase.

TABLE IV. SUBSTRATES OF MOLD MALTASE AND SACCHAROGENIC AMYLASE

Time of hydrolysis	crystalline maltase			saccharogenic amylase		
	maltose	phenol-α-glucoside	methyl-α-glucoside	maltose	phenol-α-glucoside	methyl-α-glucoside
hours,	%	%	%	%	%	%
0.5	62	±	0	21	0	0
1		4	0		0	0
18		63	3		0	0

0.5% enzyme sol: 1.5 ml., substrates: 0.05 M-3 ml.; acetate: 8 ml; Total 17.5 ml. pH: 3.4 for maltase, 4.1 for sacch. amylase; temp: 40 C

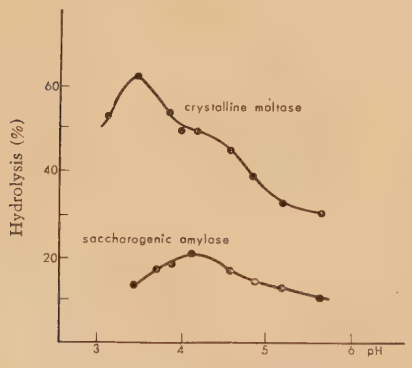

FIG. 2. Activity-pH Curves of Maltase and Saccharogenic Amlase.

0.5% enzyme 0.5 ml., 2.3% maltose 2 ml., acetate (0.5 M) 5 ml; Total 12.5 ml: 37°C, 30 min.

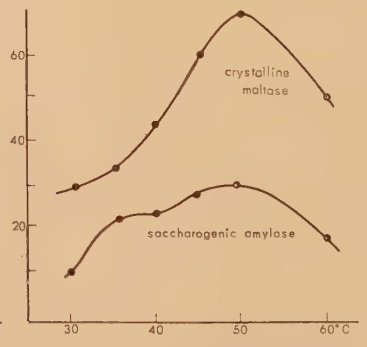

FIG. 3. Activity-Temperature Curves of Maltase and Saccharogenic Amylase.

pH: 3.4 for maltase, 4.1 for sacch. amylase. Contents of the incubation flasks are the same as Fig. 2. 20 min.

Crystalline maltase hydrolyzes maltose and phenol-α-glucoside but not appreciably methyl-α-glucoside. Hydrolysis of phenol-α-glucoside is markedly slower than that of maltose, but both substrates are hydrolyzed completely when concentration of the enzyme is high. Subsequently, mold maltase, different from yeast maltase, is not inhibited by the hydrolysis product,—glucose. Opt. pH is 3.4 (Fig. 2) and opt. temp. 50°C (Fig. 3).

A comparison of the both activities of maltose hydrolysis in mold, as shown in Table IV and Fig. 2, indicates that there are considerable differences in substrate-specificity and pH optima. Phenol- and methyl-α-glucoside are not hydrolyzed by saccharogenic amylase, and the opt. pH of this enzyme is 4.1. However, the opt. temp. is the same in both enzymes.

The authors are indebted to Mr. Masatiyo Sato of Sapporo Shyusei Kogyo Co. Ltd., for kindly supplying the "Koji" material used in this work.

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Effects of 2,4-Dinitrophenol on Endogenous Respiration of Yeast Harvested during the First Budding Cycle

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Received August 11, 1958

The first budding cycle of a bakers' yeast was synchronized with the aid of the method of starving pre-cultures. Immediately after inoculation for the synchronous culture, stimulation of endogenous respiration with 2,4-dinitrophenol (DNP) was observed, which however, disappeared when budding cycle began. During the budding cycle, inhibition effects became more stronger as the bud forming proceeded. The substrates which were dissimilated stimulative by DNP were trehalose and acid-soluble glycogen. The former was more sensitive to the increase of the concentration of DNP and was consumed by 3×10^{-4}M of DNP almost wholly, whereas the latter contained a labile part which was dissimilated stimulatively at a low concentration (less than 6×10^{-5}M).

Though many reports have appeared on the effects of 2, 4-dinitrophenol (DNP) on endogenous metabolism of yeast, the results, however, are not always similar. For example, Sheffner et al.[1] reported that DNP inhibited the endogenous respiration of *Saccharomyces cerevisiae* strain K. Simon[2] obtained the same results showing that nitrophenols suppressed endogenous respiration of bakers' yeast to the order of one-ninth that of normal respiration. On the other hand, stimulation effects were observed by Stickland[3], Rothstein et al.[4] in endogenous respiration, and by Rothstein et al.[5], Brady et al.[6] in endogenous fermentation. It is obvious that these contrasted results depend on age of culture, difference of strain, or concentration of the inhibitor, etc. In this paper results show that

1) A. L. Sheffner and D. O. McClary, *Arch. Biochem. Biophys.* **57**, 401 (1955).
2) E. W. Simon, *J. Exptl. Botany*, **4**, 377 (1953).
3) L. H. Stickland, *Biochem. J.*, **64**, 498 (1956).
4) A. Rothstein and H. Berke, *Proc. Soc. Exptl. Biol. Med.*, **81** 559 (1952).
5) A. Rothstein and H. Berke, *Arch. Biochem. Biophys.*, **36**, 195 (1952).
6) T. G. Brady and D. F. Duggan, Congr. intern. biochem. Resumés communs. 3e congr. Brussels (1955).

when a bakers' yeast cultured synchronously was harvested at various stages during its first budding cycle, washed and resuspended in buffer solution, stimulation or inhibition of endogenous respiration is dependant on the stages of the budding cycle. Namely, before the onset of budding, there was a simulating effect and during budding, inhibition occured. When stimultation was observed, trehalose and a part of acid-soluble glycogen were most labile substrates. These coincide with the result obteined by Berke et at.[7] who have shown the fact in endogenous fermentation. However, for these two fractions, the labilities by the changes of the inhibitor concentration were not the same.

Recently a few methods concerning synchronous culture of yeast have been reported[8~13]. In this paper a simple method for the first budding cycle synchronization is also presented.

MATERIALS AND METHODS

Strain: The bakers' yeast, which rapidly decreased in its bud ratio at the stationary phase, was selected and isolated from commercial bakers' yeasts.

Culture medium: Kôji-agar for stock culture slant and White-Mase medium[14] for pre- and synchronous cultures were used.

Culture method: (1) First pre-culture. In 10 ml. of the medium one loopful of stock culture was transferred and incubated at 30°C for 48 hr. (2) Second pre-culture. The first pre-culture was transferred into a 500 ml.–Erlenmeyer flask containing 100 ml. of the medium and shaken with a rotary shaker (100 r.p.m.) at 30°C for 48 hr. At the end of this incubation the bud ratio became lower than 3%. Cells were separated from the medium and washed twice with distilled water. (3) Third pre-culture. In 200 ml. of the medium in a 1 l.–Erlenmeyer flask, cells of the second pre-culture were transferred at the concentration of about 10^8 cells/ml. and aerated vigorously at 30°C. After about 6 hours glucose in the medium was consumed and a stationary phase appeared, 0.02 g. of glucose per g. of flesh yeast per hr. was added according to Hayashibe's method[11,12]. After the bud ratio became lower than 2%, glucose addition was stopped and the cells were starved for 8 hours, then harvested and washed three times with distilled water. (4) Synchronous culture. Cells of the third pre-culture were transferred into a 1 l.–Erlenmeyer flask containing 100 ml. of the medium at a concentration of about 2.5×10^7 cells/ml. and aerated at 30°C. Twenty to thirty min. was required from inoculation to the onset of budding, and the first budding cycle was accomplished within 100–120 min. (see the results)

Manometric method: Conventional manometric techniques were employed using a Warburg apparatus at 30°C. All flasks contained 0.5 ml. of buffer solution (pH 4.5, M/20 phthalate) and 0.5 ml. of cell suspension (5–10 mg. of dry weight) in the main compartment, 0.4 ml. of water or DNP in the side arm, and 0.2 ml. of 30% KOH and 2 cm^2 of filter paper in the center well. The atmosphere here was always air.

Paper chromatogram of non-reducing sugar: Solvent: *n*-butanol 6: pyridine 4: H_2O 3 (v/v). The dried, developed paper chromatogram strip was sprayed with 2% $KMnO_4$ in 3% Na_2CO_3. After drying, Trevelyan's reagent[15] was applied. With the $KMnO_4$ reagent, non-reducing sugar, such as trehalose, showed a much clearer spot than the original Trevelyan's method.

Fractionation and determination of cellular carbohydrates: Trevelyan's method[16] was used.

Cell and bud counts: Direct counting of cells and buds was carried out under a phase contrast microscope equipped with a Thomas haemacytometer or on the usual deck glass when only the bud ratio was counted.

RESULTS

Synchronous culture: Fig. 1 shows the third pre- and synchronous cultures. In the latter, about 90% of whole cells were budding after 80 min. At the commencement of the third pre-culture, the cells transferred therein possessed an extremely low bud ratio as the second pre-culture itself had a considerable long

7) H. L. Berke and A. Rothstein, *Arch. Biochem. Biophys.*, **72**, 380 (1957).
8) M. Ogur, S. Micker and D. O. McClary, *J. Bacteriol.*, **66**, 642 (1953).
9) A. Campbell, *J. Bacteriol.* **74**, 559 (1957).
10) C. A. Beam, R. K. Mortimer, R. G. Wolf and C. A. Tobias, *Arch. Biochem. Biophys.* **49**, 110 (1954).
11) M. Hayashibe, Anual meeting of Agr. Chem. Soc. Japan. 1957 at Tokyo.
12) M. Hayashibe, Anual Meeting of Agr. Chem. Soc. Japan. 1958 at Kyoto.
13) A. Campbell, *Bacteriol. Review*, **21**, 263 (1957).
14) Y. Mase, *Vitamins* (*Kyoto*) **7**, 374 (1954).
15) W. E. Trevelyan, *Nature*, **166**, 444 (1950).
16) W. E. Trevelyan, and J. S. Harrison, *Biochem. J.*, **63**, 23 (1956).

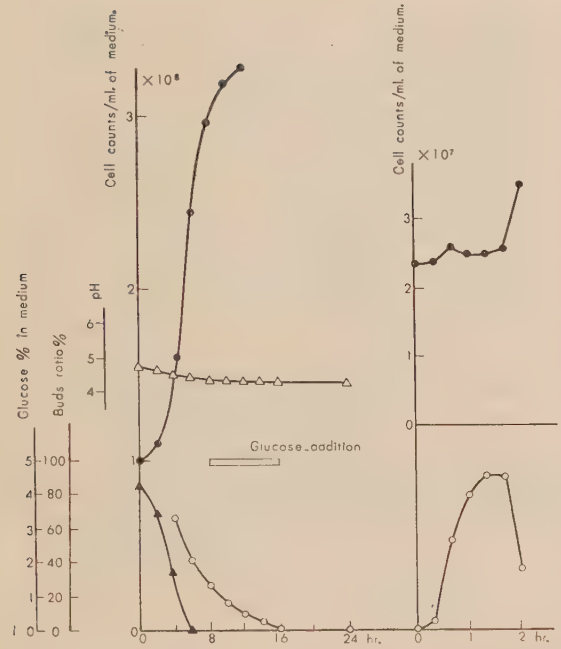

FIG. 1. The Third Pre-(left) and Synchronous (right) Culture of the Yeast.

In both, at 30 C. Glucose added is 0.02 g./g. of flesh yeast weight/hr.
-▲- glucose, -△- pH, -●- cell counts, -○- buds ratio

starvation period, so that the bud ratio was usually less than 3% and all cells were sufficiently starved. Consequently, when these cells were transferred into the third flask in a state of considerable high yeast counts, equivalent to approximately one-third of the possible yeast yield from the medium, all cells split out only one, or at most two, daughter cells prior to complete consumption of nutrients, so that the buds forming ability between each cell at the onset of the synchronous culture might be almost the same.

Effects of DNP on endogenous respiration of the yeast harvested at various stages of the first budding cycle: From the onset of synchronous culture the cells were harvested every 30 min. and washed three times with cold distilled water, and the endogenous respiration was measured manometrically with and without 3×10^{-4} M of DNP (Fig. 2). It was considered that during this measurement the growth of the cells (except the fully starved 0 min.-cells) might occur by using their endogenous materials. The bud ratio before and after this measurement, however, was unchanged. Strictly speaking, even if the bud ratio was unchanged, the growth of the cells might be expected. In this paper, however, interest is chiefly directed to the contrasted results refferred above considering whether the effects of DNP are dependant on the

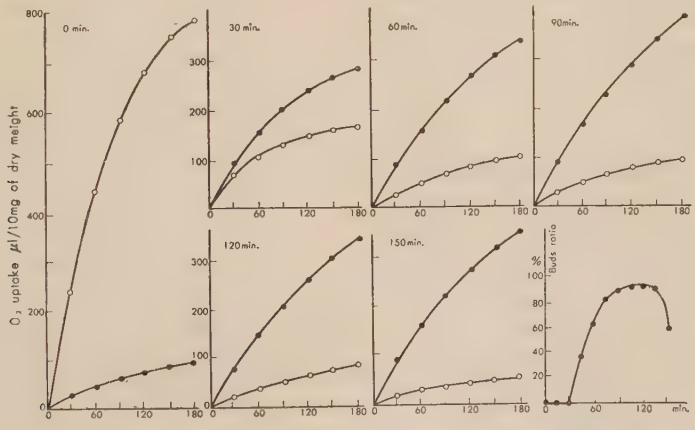

FIG. 2. The Fffects of DNP on the Yeast Endogenous Respiration.

The first budding cycle of the yeast is also shown. Time specified in upper left corner of each figure shows the stage at which the yeast are harvested. All the experiments were measured at 30°C.
-●- without DNP, -○- with DNP (3×10^{-4} M)

harvested stages or not. At this point of view, these results were analized. Prior to budding, DNP stimulated on the order of seven times that of net endogenous respiration. However, at the onset of budding it began to inhibit endogenous respiration and inhibition became stronger, finally resulting immediately before the end of the budding cycle on the order of one-seventh that of net-endogenous respiration. These result would seemingly indicate that, at zero min., cells possessed reserve carbohydrates which were able to be consumed stimulately with DNP, but as budding proceeded these reserves were consumed for the de novo synthesis of cell materials or for another purpose; consequently, subsequent to the beginning of budding, cells were already devoid of such materials. On the other hand, some other substrates (or substrate) of net endogenous respiration were increasing during budding, and if the oxidation of these substrates were to be inhibited with DNP, apparent inhibition would be observed. It may be interesting to investigate the kind of cellular materials which are consumed so rapidly in the presence of DNP and show a large amount of Oxygen uptake. Stickland[3] showed that the substrates were cellular carbohydrates in aerobic condition, and recently Berke et al.[7] also reported that under the anaerobic condition trehalose was more mobile than any other cellular carbohydrates. Hence, the stimulation effect was investigated by using the zero min. cells.

CO_2 production and O_2 uptake in the presence of DNP: O_2 uptake and CO_2 production showed (Fig. 3) a maximum value and respiratory quotient (R.Q.) of one with 10^{-4} M of DNP after 180 min.-incubation in Warburg flasks. However, at high concentration, R.Q. was higher than one, while O_2 uptake and CO_2 production decreased. For example, at 10^{-3} M, the observed R.Q. was about three. This shows that at such concentration of DNP non-respiratory CO_2 production takes place. On the other hand, changes of cellular carbohydrates before and after this experiment showed (Fig. 4) that at a lower concentration of DNP (less than 6×10^{-5} M) some part of glycogen, especially of acid-soluble glycogen, was

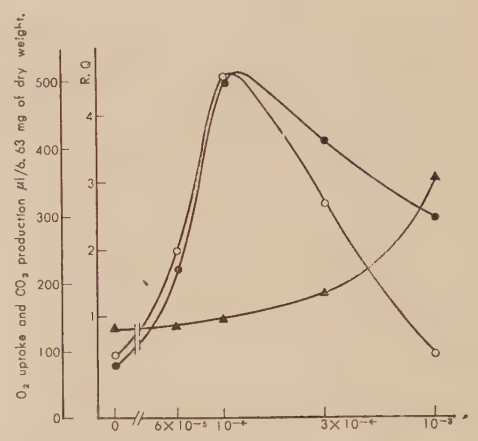

FIG. 3. Stimulating Effect of DNP on Endogenous Respiration of Starved (zero min. cell) Yeast.
O_2 uptake and CO_2 production for 180 min. at 30°C.
-●- CO_2, -○- O_2, -▲-R.Q.

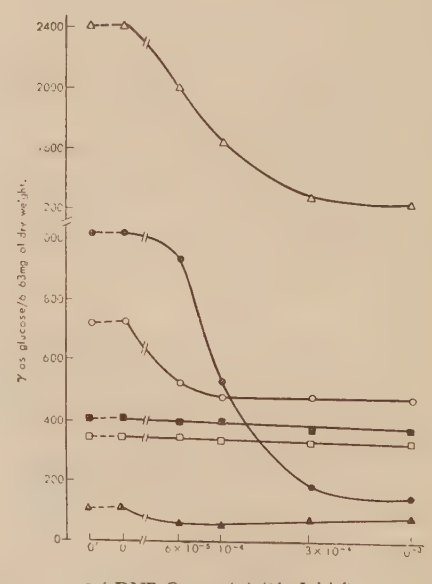

FIG. 4. Carbohydrate Content of the Starved Yeast before and after the Measurement of Stimulating Effect Illustrated in Fig. 3.
-△- Total carbohydrate, -●- Trehalose, -○- Acid-sol. glycogen, -▲- Alkali-sol. glycogen, -■- Glucan, -□- Mannan

dissimilated more easier than trehalose (M/2 cold trichloroacetic acid soluble fraction), while at higher concentrations of DNP (exceeding 10^{-4} M) trehalose dissimilation dominated and acid-soluble glycogen was insensitive to the changes of concentration of DNP. Net endogenous respiration (zero M of DNP) did not require any part of cellular carbohydrates. Mannan and glucan kept at a constant level and showed no decrease due to any concentration of DNP.

From these two data the following conclusion may be made:

DNP effects on starved yeast were classified into three types according to its concentration:

1) At lower concentrations (less than 6×10^{-5}M), some parts of acid-soluble glycogen and a less amount of trehalose were dissimilated and R.Q. was below unity.

2) At the medium concentrations (6×10^{-5}M), trehalose was dissimilated dominantly and R.Q. was about one.

3) At higher concentrations (exceeding 10^{-4}M), trehalose dissimilation dominated R.Q. was higher than one, whereas the glycogen which had escaped from dissimilation at lower concentration was also not consumed under this concentration.

Paper chromatogram of TCA soluble fraction of the yeast: Trevelyan[17,18] has proved that the yeast carbohydrate extracted with 10% or M/2 of trichloroacetic acid and showing anthrone positive was trehalose by application of paper partition chromatography. These results were re-examined using a slightly modified method (see item of method). There was no sugar except trehalose in this fraction.

Effects of DNP on endogenous respiration of the budding cells: As the above data shows, DNP stimulated oxidation of trehalose and a part of acid soluble glycogen. It was, therefore, examined whether the budding yeast cells had such substances or not. The changes of carbohydrates during the first budding cycle is

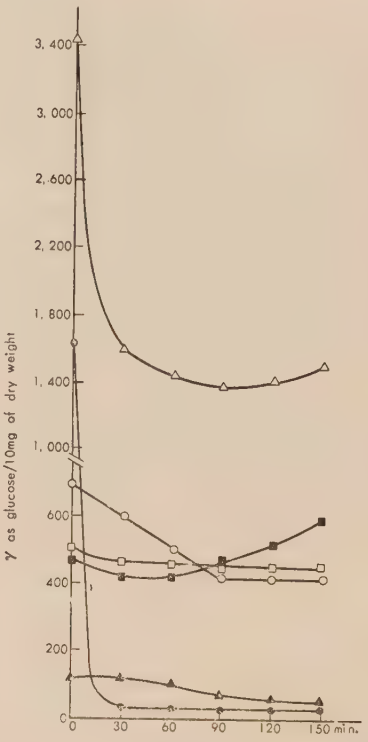

FIG. 5. The Carbohydrates of the Yeast during the First Budding Cycle in Full Medium.
-△- Tatal carbohydrate, -●- Trehalose, -○- Acid sol. glycogen, -▲- Alkali-sol. glycogen, -■- Glucan, -□- Mannan.

shown in Fig. 5 by using the same yeast as used in the experiment shown in Fig. 2. During the first 30 min. total carbohydrate decreased rapidly, and a rapid decrease of trehalose in cells was also observed. This coincided with the results obtained by Brandt[19] and Pollock[20]. After the beginning of the budding cycle, trehalose showed no further increase but remained at an extremely low level; while, on the other hand, changes in the acid-soluble glycogen were remarkable. A gradual decrease was observed for the first 90 min., however, following this period, it still maintained a considerable high level; as for example, about 1/3.5 of the total carbohydrate at 90 min. was the acid-soluble glycogen. This result demonstrates

17) W. A. Trevelyan and J. S. Harrison, *Biochem. J.*, **50**, 298 (1952).
18) W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, **62**, 177 (1956).
19) K. M. Brandt, *Biochem. Z.*, **309**, 190 (1940).
20) G. E. Pollock and C. D. Holmstrom, *Cereal Chem.*, **28**, 498 (1951).

that the cells of which endogenous respiration was inhibited with DNP still contained a considerable amount of the glycogen. Hence, by using this cell the same experiment as that on starved cells was performed. As already shown, the endogenous respiration of the cells were inhibited with 3×10^{-4} M of DNP. Moreover, the measurement of carbohydrates prior to and after observation of gas exchange showed (Table I) that the remaining acid-soluble glycogen or any other carbohydrates were not stimulatively dissimilated with the DNP treatment.

TABLE I. O_2 UPTAKE AND CO_2 PRODUCTION, AND CARBOHYDRATES CONTENT OF THE BUDDING YEAST CELL (harvested at 90 min. during the first budding cycle) PRIOR TO AND AFTER ENDOGENOUS RESPIRATION (180 min.) IN THE PRESENCE AND ABSENCE OF DNP (3×10^{-4}M). (per 10.7 mg. of dry weight.)

	Initial	With DNP	Without DNP
O_2 uptake (μ l.)	—	68	287
CO_2 production (μ l.)	—	92	240
Total	1481	1495	1475
External	0	4	5
Trehalose	48	22	21
Mannan	481	481	468
Alkali-sol. glycogen	38	50	65
Acid-sol. glycogen	443	454	425
Glucan	471	484	491

(γ. as glucose)

It was thus concluded that the acid-soluble glycogen remaining after the onset of the budding was not metabolically labile. Fales[21] has reported on a metabolically labile, alkali-insoluble carbohydrate (transient carbohydrate) in freshly fed yeast. This might coincide with the labile part of the acid-soluble glycogen in Figs. 4 and 5. Mannan and glucan were metabolically inert in either the presence or absence of DNP.

DISCUSSION

Of the five carbohydrates fractions studied in this paper, glucan and mannan were inert from a metabolic point of view. There was not any mobilization of these fractions by DNP. However, glucan, which is known as a constituent of the yeast cell wall, was apparently synthesized during the budding cycle. In the remaining three fractions, acid-soluble glycogen and trehalose served as a labile carbohydrate storage depot, decreasing markedly during the budding cycle or in the presence of DNP. But these were not labile during starvation (for 180 min. at 30°C), coinciding with the results of Stickland[3]. Alkali-soluble and insoluble glycogen have been reported to be similar chemically[17,22]. In the present data those two fractions showed the same behavior during the budding cycle or in the presence of DNP. There was, however, a difference in response of trehalose and acid-soluble glycogen to DNP; firstly, when using sufficiently starved non-bud cells, at a lower concentration of DNP, the glycogen fraction was more mobile than trehalose; on the other hand, at higher concentrations, this relation was reversed, finally at the highest, trehalose decreased at a markedly high R.Q. (about three), whereas the some part of the glycogen maintained a constant level. As to glycogen, a similar relation was observed during the budding cycle. During the first 30 min. trehalose decreased very rapidly, against glycogen which dereased only slowly, of which even after 90 min., a considerable amount remained. From these it may be concluded that the acid-soluble glycogen might be heterogeneous, at least from the physiological view.

The stimulating and inhibiting effects of DNP on the endogenous respiration of yeast, which have been reported by many authors not always obtaining the same results, were considered to be as follows; Prior to the onset of the budded cycle, i.e., before the growth and division of the cell, cells (sufficiently starved) possess a considerable amount of DNP sensitive reserve carbohydrates, especially trehalose and a part of acid-soluble glycogen. Thus stimulation is observed. However, at the onset of and during the budding cycle, these carbohydrates have

21) F. W. Fales, *J. Biol. Chem.*, **193**, 113 (1951).

22) M. R. Steteen and D. Steteen, Jr., *J. Biol. Chem.*, **207**, 331 (1954).

heen consumed and a substrate (or substrates) of newly accumulated endogenous respiration which are inhibited by the DNP gradually appear in the cells, so that inhibition might be observed.

The R.Q. of stimulated endogenous respiration depended on the concentration of DNP. Under lower concentrations, it assumed a value of one, whereas at higher concentrations, it showed values higher than one. The high values, which Stickland[3] had observed, would be caused by the non-respiratory CO_2 production of DNP-sensitive stores, especially of trehalose. Further, it would also be suggested that high concentration of DNP inhibited the O_2 uptake pathway in normal carbohydrate oxidation by the yeast.

The mechanism on the enzyme level by which DNP exerts its effects on carbohydrate storage is still not clear. Trevelyan et al.[23] after studying a similar action of azide suggests that the inhibitor exerts its effects by blocking phosphorylation thus increasing the orthophosphate level in the cell. It is also interesting to observe the suggestion of Berke et al.[7] that DNP activates trehalase within the cell, or perhaps DNP activates the phospholysis of trehalose to glucose plus glucose phosphate.

23) W. E. Trevelyan, J. N. Gammon and J. S. Harrison, *Biochem J.*, **50**, 303 (1951).

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 165~171, 1959]

Pseudomonas Pigments. IV.

The Structnre of Pyoluteorin*

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Received August 25, 1958

Degradation studies on pyoluteorin have been conducted. Alkali fusion of dechloro-O, O′, N-methylpyoluteorin (II) afforded 1,3-dimethoxybenzene and N-methylpyrrole-2-carboxylic acid. Lithium aluminum hydride reduction of II gave 2,6-dimethoxyphenyl-N-methyl-2′-pyrryl-methanol (VI) indicating that a carbonyl group is linked directly to both the benzene and the pyrrole ring. Further, chromic acid oxidation of II furnished N-methyl-2,6-dimethoxybenzoylformamide (VII) which was also obtained from O, O′, N-methylpyoluteorin (I). Alkaline hydrolysis of VII gave the corresponding keto acid which was then converted with hydrogen peroxide to 2,6-dimethoxybenzoic acid (IX). In view of these results, pyoluteorin must have the constitution of 2′-(2,6-dihydroxybenzoyl)-x,x′-dichloropyrrole.

As a continuation of work on the structure of a pigment, pyoluteorin[1,2], produced by *Pseudomonas aeruginosa*, T 359, degradation studies of the pigment and its derivatives have been carried out. All attempts to obtain degradation products of the pigment resulted unsuccessful. However, degradation of O, O′, N-methylpyoluteorin $C_{11}H_4ONCl_2(CH_3)(OCH_3)_2$ (I) and dechloro-O, O′, N-methylpyoluteorin $C_{11}H_6ON(CH_3)(OCH_3)_2$ (II), described previous-

* A preliminary report of this investigation has been published in the *J. Am. Chem. Soc.*, **80**, 4749 (1958).

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1) R. Takeda, *J. Ferm. Tech., Osaka*, **36**, 281 (1958).

2) R. Takeda, This Bulletin, **23**, 126 (1959).

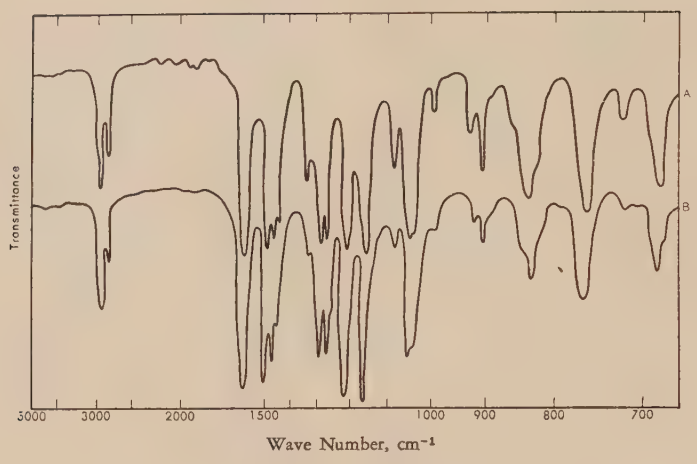


FIG. 1. Infrared Spectra, liquid, 0.01 mm thick
A, III; B, Authentic 1,3-Dimethoxybenzene.

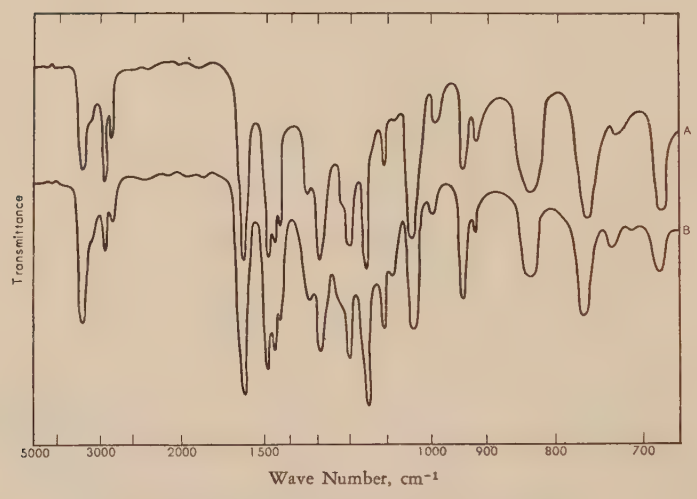


FIG. 2. Infrared Spectra, liquid, 0.01 mm thick
A, IV; B, Authentic 1-Methoxy-3-hydroxybenzene.

ly[2], proceeded satisfactorily giving several compounds which aided in the determination of the constitution. Although hydrolysis with alkaline solution or zinc dust distillation of II yielded no definite products except the starting material; II on hydrolysis with 20% hydrochloric acid gave oily products which were separated into two parts by fractionating with 10% sodium hydroxide and ether. From the ether fraction was yielded a pale yellow distillate, $C_8H_{10}O_2$ (III) which was identical with an authentic 1, 3-dimethoxybenzene[3] (Fig. 1). The distillate $C_7H_8O_2$ (IV) from the alkali soluble part gave the same absorption pattern in infrared spectrum with that of an authentic sample of 1-methoxy-3-hydroxybenzene[3] (Fig. 2). Since both of these substances can arise from the same half of the pyoluteorin molecule according to conditions of the experiment, the nitrogen atom

3) F. Tiemann and A. Parrisius, *Ber.*, **13**, 2362 (1880).

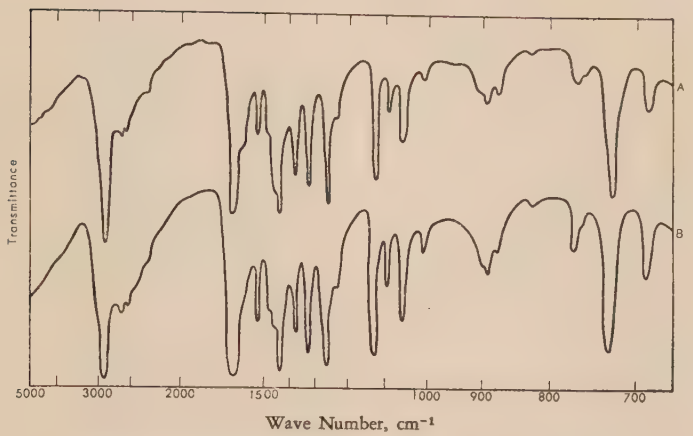
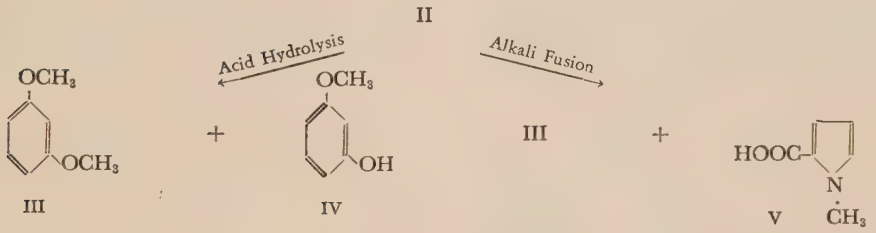


FIG. 3. Infrared Spectra, Nujol mull, 0.01 mm thick
A, V; B, Authentic N-Methylpyrrole-2-carboxylic Acid.



should be in the other half of the molecule. As was expected, the ethereal extract of the acid hydrolysis mixture gave a positive reaction with Ehrlich's reagent to pyrrole compounds, although no definite product could be isolated.

Alkali fusion of II was then conducted to obtain compounds derivable from the other half of the molecule, as pyrrole compounds are generally stable in alkali. The acidic product $C_6H_7O_2N$ (V), which, in fact, gave coloration with Ehrlich's reagent was identical with an authentic sample of N-methylpyrrole-2-carboxylic acid[4] (Fig. 3). And, as was expected, an oily substance obtained from the neutral fraction was identified as III by infrared spectrum.

Since pyoluteorin and all of its derivatives gave an absorption in infrared spectrum around 1630 cm^{-1} indicating cross-conjugated C=O radicals as described previously[2], and besides, the disubstituted benzenes III and IV, the pyrrole derivative V having a carboxyl group at its 2-position has also been obtained from II, the only possible linkage between the benzene ring and the 2-position of pyrrole nucleus must be the carbonyl group. To confirm the ketone linkage, II was treated with lithium aluminum hydride[5] to give dimethoxyphenyl-N-methyl-2′-pyrrylmethanol $C_8H_9O_2$-CHOH-C_5H_6N (VI) which melts at 135–136° and decomposes gradually in the air to a dark purple colored substance. Although the product could not be analyzed due to its instability, the absorption gave an ultraviolet maxima at 241 mμ of a pyrrole ring and at 271 mμ of a benzene ring, but gave no maximum around 300 mμ characteristic of pyoluteorin (Fig. 4) and also showed a band at 3530 cm^{-1} for OH group in the infrared spectrum indicating no cross-conjugated C=O band at 1640 cm^{-1}

4) T. Reichstein, *Helv.*, **13**, 354 (1930).

5) M. S. Newman and N. C. Deno, *J. Am. Chem. Soc.*, **73**, 3644 (1951).

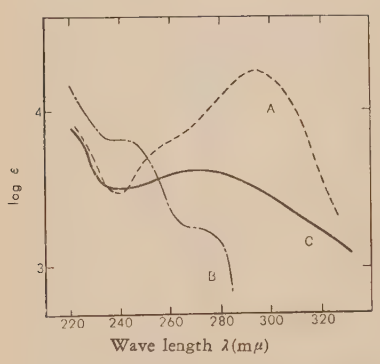


FIG. 4. Ultraviolet Absorption Spectra, Ethanol
A: II
B: VI
C: VII

being observed. Therefore it is evident that a carbonyl group is linked directly to both the benzene ring and the 2-position of pyrrole nucleus in the pyoluteorin molecule.

the ultraviolet absorption maximum at 272 mμ of benzene ring (Fig. 4) and the infrared absorption bands at 3270, 1525 cm^{-1} for the NH group and 1750, 1690, 1670 cm^{-1} for cross-conjugated C=O and CONH groups. As the same product was also obtained from I by similar treatments, it has become evident that chlorine atoms of the pyoluteorin molecule are located on the pyrrole ring and the product obtained is supposed to be N-methyl-dimethoxy-benzoylformamide. VII was then hydrolyzed with aqueous potassium hydroxide to the corresponding keto acid (VIII) which, without being isolated was further converted with hydrogen peroxide to a dimethoxybenzoic acid $C_9H_{10}O_4$, m.p. 187°. The infrared spectrum and mixed melting point determination showed the above acid to be identical with 2,6-dimethoxybenzoic acid (IX) among the three isomeric dimethoxy-

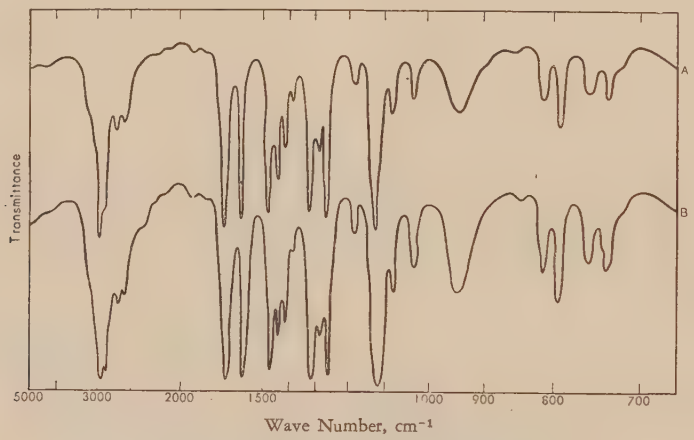
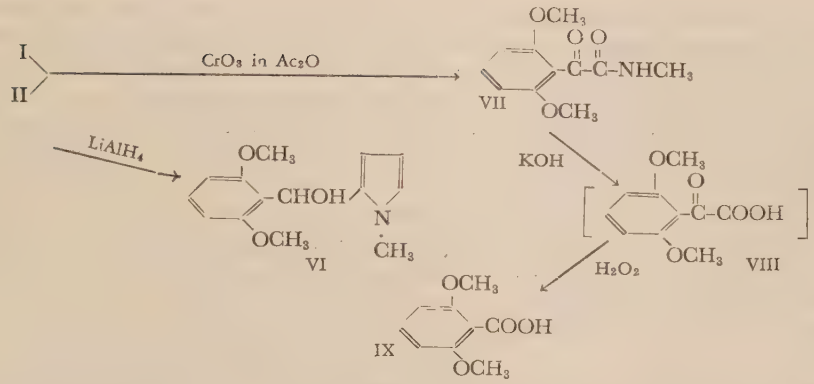


FIG. 5. Infrared Spectra, Nujol mull, 0.01 mm thick
A, Degradation Product of VII;
B, Authentic 2,6-Dimethoxybenzoic Acid (IX).

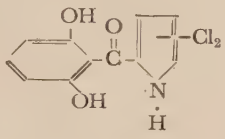
Further degradation of I and II was carried out so as to determine the position of the carbonyl group on the benzene ring as well as the position of the two chlorine atoms. Whereas, on oxidation of II with permanganate no appreciable amount of product was obtained under the conditions employed; the oxidation of II with chromium trioxide afforded colorless needles (VII) $C_{11}H_{13}O_4N$, m.p. 143°, which gave benzoic acids synthesized by known methods[6,7,8] (Fig. 5).

In view of the results mentioned above, pyoluteorin must have a constitution of 2′-(2,6-dihydroxybenzoyl)-x, x′-dichloropyrrole (X).

6) N. J. Cartwright, J. I. Jones and D. Marmion, *J. Chem. Soc.*, **1952**, 3499.
7) D. A. Clibbens and M. Nierenstein, *J. Chem. Soc.*, **107**, 1494 (1915).
8) S. Ludwinowsky and J. Tambor, *Ber.*, **39**, 4039 (1906)



As described previously[2], pyoluteorin and dechloropyoluteorin on methylation gave O, O′, N-methyl and O, O′-dimethyl derivatives respectively. This may suggest that chlorine atoms on the pyrrole ring increase the acidic nature of the NH-group of the molecule. It is therefore considered reasonable that one of the two chlorine atoms of the molecule may assume the 5′-position of the pyrrole ring.



In connection with pyoluteorin it is of interest to note that prodigiosin[9] produced by *Serratia marcescens* is also a pyrrole derivative which has been considered as a possible intermediate in biosynthesis of porphyrin, and that both strains, *Pseudomonas* and *Serratia*, have the same characteristic properties of oxidizing glucose to 2-ketogluconic acid[10].

Further studies on the position of the chlorine atoms on the pyrrole ring are now underway.

EXPERIMENTAL***

Acid Hydrolysis of Dechloro-O,O′, N-methylpyoluteorin (II).

II (2.9 g.) was heated under reflux with 20% aqueous hydrochloric acid (250 ml.) in a flask on an oil bath for one hour and a half. After cooling the reaction mixture the flocculent precipitate was filtered off and the filtrate extracted with ether. The ether solution, after washing with water and 1% sodium hydrogen carbonate solution, was extracted with 10% sodium hydroxide solution.

A) 1,3-Dimethoxybenzene (III). The ether solution of the above extraction was washed with water and dried over anhydrous sodium sulfate. After removal of the ether, the oily residue was distilled under vacuum to give a pale yellow distillate, b.p.$_{22}$ 105°–106°. $\lambda_{max.}^{EtOH}$ 274 mμ (ε 2210). 280 mμ (ε 2,072). *Anal.* Found: C, 69.76; H, 7.13. Calcd. for $C_8H_{10}O_2$: C, 69.54; H, 7.30. The product was identical with an authentic sample of 1,3-dimethoxybenzene synthesized (Fig. 1).

B) 1-Hydroxy-3-methoxybenzene (IV). The alkaline extract was acidified with 6N hydrochloric acid and extracted with ether. Removal of ether, followed by vacuum distillation yielded an oily substance, b.p.$_{25}$ 140°. $\lambda_{max.}^{EtOH}$ 274 mμ (ε 2350), 280 mμ (ε 2,300), 293 mμ (ε 1,070). *Anal.* Found: C, 68.02; H, 6.33. Calcd. for $C_7H_8O_2$: C, 67.74; H, 6.45. The product was in coincidence with an authentic 1-hydroxy-3-methoxybenzene (Fig. 2).

Alkali Fusion of Dechloro-O, O′, N-methylpyoluteorin (II).

II (0.5 g.) was heated with potassium hydroxide (5.0 g.) and water (0.5 ml.) for thirty minutes at 250°. The reaction mixture was diluted with fifty ml. of water and the solution was extracted three times with a portion of fifty-milliliters of ether.

A) 1,3-Dimethoxybenzene (III). The ether solution was washed with water and dried. After removal of the ether, the oily residue was distilled to give a pale yellow liquid, b.p.$_{23}$ 105°, *Anal.* Found: C, 69.82; H, 7.07. Calcd. for $C_8H_{10}O_2$: C, 69.54; H, 7.30,

9) R. Hubbard and C. Rimington; *Biochem. J.*, **46**, 220 (1950).
10) Y. Ikeda; *J. Agr. Chem. Soc. Japan*, **24**, 151 (1951).
*** All melting points are uncorrected; the ultraviolet absorption spectra were taken with a Beckman Model DK-2 in ethanol; the infrared spectra were measured in Nujol mulls unless otherwise stated by a Perkin-Elmer Model 21

the infrared spectrum of which was in good agreement with that of the authentic 1,3-dimethoxybenzene.

B) N-Methylpyrrole-2-carboxylic Acid (V). The aqueous layer, after being saturated with sodium chloride, was acidified with concentrated hydrochloric acid under cooling, and the resulting precipitate was extracted with ether. The ethereal solution was washed with water, and dried over anhydrous sodium sulfate. The ether was removed *in vacuo* and the amorphous residue was crystallized from hot water to give colorless needles, yield 62 mg., m.p. 135°. *Anal.* Found: C, 57.32; H, 5.42; N, 11.23. Calcd. for $C_6H_7O_2N$: C, 57.59; H, 5.64; N, 11.20.

C) Synthesis of N-Methylpyrrole-2-carboxylic Acid.

i) Ethyl N-Methylpyrrole-2-carboxylate: Ethyl pyrrole-2-carboxylate was synthesized following the method reported in the literature[4]. Grignard reaction of pyrrole (20 g.), and ethyl bromide (35 g.) and ethyl chloroformate (35 g.) gave 12.9 g. of the compound $C_7H_9O_2N$, $b.p._{0.4}$ 95–97°C. Ethyl pyrrole-2-carboxylate (7 g.) was mixed with metallic potassium (2.5 g) in toluene (50 ml.) and the mixture was heated at a temperature around 65°C. Methyl iodide (30 g.) was then added to the reaction mixture and the potassium iodide separated out was filtered off. The filtrate was concentrated *in vacuo* and the residue distilled to give ethyl N-methylpyrrole-2-carboxylate. Yield 4.5 g., $b.p._{0.6}$ 60–61°. *Anal.* Found: C, 62.69; H, 7.11; N, 8.85. Calcd. for $C_8H_{11}O_2N$: C, 62.72; H, 7.24; N, 9.14.

ii) N-methylpyrrole-2-carboxylic Acid: Ethyl N-methylpyrrole-2-carboxylate (3.5 g.) was mixed with potassium hydroxide (13 g.) in water (65 ml.) containing a small amount of ethanol and the mixture was heated for four hours at 150°. After cooling and washing with ether, the reaction mixture was acidified under cooling with ice water. The precipitate was extracted with ether. The ethereal solution was treated in a conventional way to yield N-methylpyrrole-2-carboxylic acid which was crystallized from hot water in colorless needles, yield 1.9 g., m.p. 135°[11][12]. *Anal.* Found: C, 57.45; H, 5.53; N, 11.14. Calcd. for $C_6H_7O_2N$: C, 57.59; H, 5.64; N, 11.20.

Reduction with Lithium Aluminum Hydride. Dimethoxyphenyl-N-methyl-2′-pyrrylmethanol (VI).

To a solution of lithium aluminum hydride (1.0 g) in anhydrous ether (100 ml.), was added II (0.5 g.). The solution was refluxed for five hour with stirring. After adding water to decompose the excess lithium aluminum hydride, the ether layer was washed and dried. The product was recrystallized from ether to yield colorless needles which gradually decomposed to a colored substance, m.p. 135–136°. $\lambda_{max.}^{EtOH}$ 241 mμ (ε 6,600) for a pyrrole ring, 271 mμ (ε 1,800) for a benzene ring. ν(OH) 3530 cm^{-1}, no conj. C=O band, not analyzed due to its instability.

Oxidation with Chromium Trioxide in Acetic Acid.

A) N-Methyl-dimethoxybenzoylformamide (VII).

i) From Dechloro-O,O′,N-methylpyoluteorin (II): II (1.0 g.) was dissolved in glacial acetic acid (150 ml.) and 80% acetic acid (96 ml.) containing chromium trioxide (3.0 g.) was added dropwise with mechanical stirring at room temperature covering a period of thirty minutes. The mixture was warmed on a water bath at 55–60° for two hours and ethanol was subsequently added to decompose the excess chromium trioxide. After evaporation, the residue was dissolved in water (70 ml.), and the solution was extracted with eight successive lots of fifty ml. of ether. After concentrating the solution to a volume of one hundred ml. and extracting the concentrate with six lots of 2% sodium hydroxide (10 ml.), the ether solution was treated in the usual manner to yield the product which was recrystallized from ether in colorless needles, yield 480 mg., m.p. 143°. $\lambda_{max.}^{EtOH}$ 272 mμ (ε 4,200) (Fig. 4); ν(NH) 3270, 1525 cm^{-1}, ν (cross-conj. C=O and CONH) 1705, 1690, 1670 cm^{-1}. *Anal.* Found: C, 59.34; H, 5.79; N, 6.33. Calcd. for $C_{11}H_{13}O_4N$: C, 59.18; H, 5.87; N, 6.28.

The combined alkaline extracts were acidified with hydrochloric acid and extracted with ether. The amount of the product obtained from this fraction was too small that it could not be identified.

ii) From O,O′,N-methylpyoluteorin: I (2.0 g.), when treated in the same way, gave the same product as in the oxidation of II, yield 400 mg., m.p. 142°, their identity being shown by mixed melting point and infrared spectra.

B) Dimethoxybenzoic Acid. VII (0.8 g.) was refluxed with 10% potassium hydroxide for two hours during which the evolution of amine was observed. The reaction mixture, after being washed with ether and two ml. of hydrogen peroxide was added thereto and kept for one hour at room temperature and then washed with ether. The aqueous layer after being acidified with hydrochloric acid, was extracted with ether and the ether solution was washed with water and dried. After removing the ether, the product was recrystallized twice from benzene to yield colorless needles, yield

11) C. A. Bell, *Ber.*, **10**, 1866 (1877);
12) E. Fischer, *ibid.*, **46**, 2510 (1913)

200 mg, m.p. 186–187°. *Anal.* Found: C, 59.26; H, 5.44. Calcd. for $C_9H_{10}O_4$: C, 59.33; H, 5.53.

C) Synthesis of 2,6-Dimethoxybenzoic Acid (IX). *m*-Dinitrobenzene (83 g.) was treated with potassium cyanide in methanol to yield 2,6-dimethoxybenzonitrile (13.5 g.). The nitrile was refluxed in concentrated potassium hydroxide solution and the resulting potassium salt treated with concentrated hydrochloric acid to yield 2,6-dimethoxybenzoic acid, yield 14 g., m.p. 186°. *Anal.* Found: C, 59.28; H, 5.39. Calcd. for $C_9H_{10}O_4$: C,59.33; H, 5.53.

The author wishes to thank Dr. K. Sato, Director of this Institute and Dr. S. Kuwada, Director of the Reasearch Laboratories, Takeda Pharmaceutical Industries, Ltd., Prof. E. Funakubo, Prof. S. Teramoto and Prof. G. Terui of Osaka University, and also Prof. L. Anderson of University of Wisconsin for their interest and encouragement of the problem. Thanks are also due to Dr. M. Abe of this Institute, Dr. H. Nawa, Dr. T. Kanzawa and Mr. M. Nishikawa of the Research Laboratories, Takeda Pharmaceutical Industries, Ltd., for their courtesies extended throughout this work, and Mr. I. Nakanishi, Miss. F. Okitsu and Miss. H. Ichioka for their cooperation in carrying out the experiments.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 171～174, 1959]

Relationship between Stereoisomerism and Biological Activity of Pyrethroids

Part IV. The Oxidative Degradation of (±)-Allethrolone-methylether*

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Received September 1, 1958

(±)-Allethrolone-methylether (II), when first oxidized by ozone and then by potassium hypobromite, affords (±)-α-methoxysuccinic acid (V) through the intermediate keto acids (III) and (IV).

In connection with the elucidation of the absolute configuration of naturally derived pyrethrolone and cinerolone, a preliminary study was made with readily accessible (±)-allethrolone in order to gain information whether it affords some potential key intermediate of the well-established configuration by chemical conversions. The oxidative degradation of free allethrolone which would lead to malic acid in the sequal was rejected because of the experimental difficulty to be predicted by the feeble rotatory power of malic acid*** which would result from the analogous oxidations of optically active pyrethrolone and cinerolone. Thence, the authors' attention was directed to allethrolone-methylether which in the sequel of appropriate oxidations would

* Part III: This Bulletin, **22**, 393 (1958)

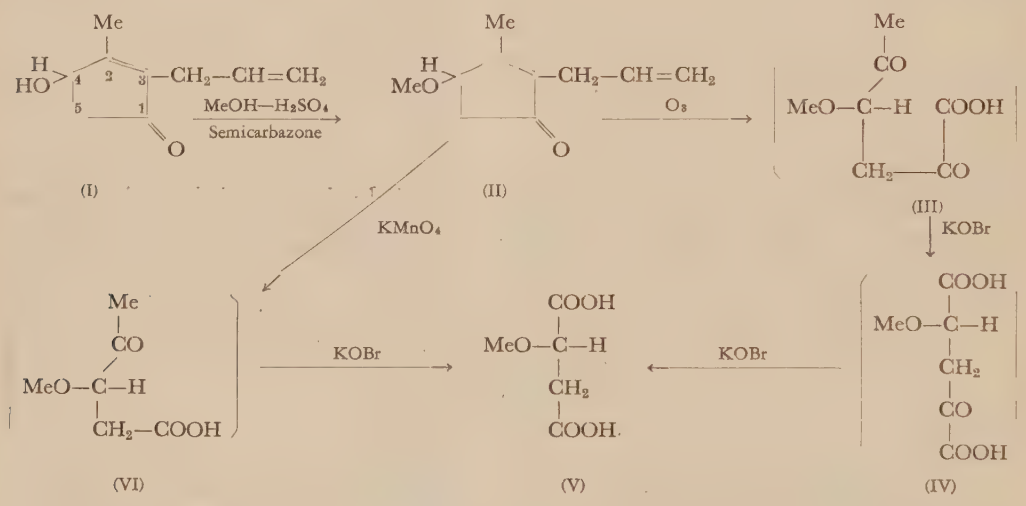
** Institute for Chemical Research, Kyoto University.

*** Stereochemical correlations between malic acid, α-methoxysuccinic acid and glyceraldehyde will be detailed in the next paper which deals with the oxidations of naturally derived pyrethrolone and cinerolone.

afford α-methoxysuccinic acid which, in the case of optically active isomer, has a high rotation sufficient to permit reliable observations.

(±)-Allethrolone-methylether (II) was prepared by procedures which Staudinger and Ruzicka[1] adopted in the preparation of pyrethrolone-methylether.

(±)-Allethrolone-semicarbazone was converted by boiling in methanol with a small amount of sulfuric acid, into the corresponding methylether, b.p. 72–3°/2 mm., in a satisfactory yield. The replacement of the semicarbazone by free allethrolone reduced the yield of the methylether desired.

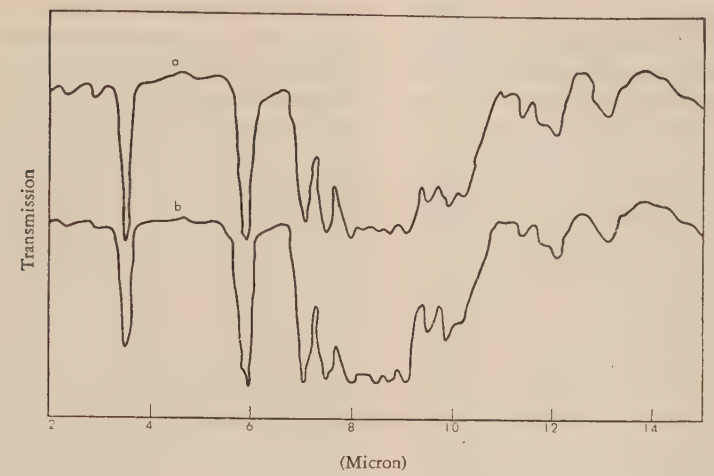


Concerning the oxidation of *cyclo*pentenolones and the related compounds, scanty references are available. Staudinger and Ruzicka[1] isolated malonic acid and an acetoxyacid from the ozonolysis of pyrethrolone acetate, capric acid, from the permanganate oxidation of tetrahydropyrethrolone while they obtained capric, laevulinic and succinic acids from tetrahydropyrethrone by permanganate oxidation.

Then, allethrolone-methylether was subjected to stepwise oxidations. Ozonolysis of allethrolone-methylether gave, in addition to neutral products, a complex mixture of acid. After the removal of volatile formic and ether-insoluble oxalic acids, the acid fraction consisted mainly of a keto-acid of the structure (III), which, though not isolated pure, was identified by its positive reaction against carbonyl reagents as well as by the formation of bromoform in the subsequent hypobromite oxidation. When the crude keto-acid (III) was oxidized with potassium hypobromite, it consumed ca. 6 mols of the oxidants and yielded bromoform together with a dicarboxylic acid which after recrystallizations, melted at 104–5°. The elemental analysis, methoxy-determination and equivalent weight by titration confirmed this acid to be α-methoxysuccinic acid. This was also characterized by the identity in every respect with the authentic specimen.

The formation of bromoform and the mols of hypobromite consumed suggest that this oxidation proceeds through an intermediate α-methoxy-α'-ketoglutaric acid (IV). An alternative oxidation sequence also afforded the same end product (V). The permanganate oxidation of allethrolone-methylether gave a mixture of acid, from which volatile and ether-insoluble acids were removed to leave a keto-acid fraction, although this fraction was not isolated pure, its structure was considered to be (VI) because it yielded a 2, 4-dinitrophenyl-

1) H. Staudinger and L. Ruzicka, *Helv.*, **7**, 212 (1924).



Infra-red spectra of a) (±)-α-methoxysuccinic acid dimethylester (liquid), b) synthetic specimen (liquid)

hydrazone m.p. 105° and consumed 3 mols of potassium hypobromite to give (V) together with bromoform.

The well-demonstrated *cyclo*pentenolone structure of allethrolone and consequently of its methylether necessitates that the carbon skeleton of the resulting α-methoxysuccinic acid represents the fragmental $C_{(3)}$–$C_{(4)}$–$C_{(5)}$–$C_{(1)}$ chain in this order in the parent compound.

This chemical conversion of allethrolone-methylether to α-methoxysuccinic acid should render it feasible to establish, by analogous oxidations of (+)-pyrethrolone- and (+)-cinerolone methylethers, the chemical correlation of naturally derived pyrethrolone and cinerolone with glyceraldehyde *via* the key intermediate α-methoxysuccinic acid.

EXPERIMENTAL

Melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division of Mitsui Laboratory of Kyoto University, to whom the authors' thanks are due. Infrared spectra were recorded with a Parkin-Elmer double beam spectrophotometer Model 21.

Allethrolone-methylether

Allethrolone semicarbazone (25g. m.p. 211–213°, La Forge et al.[2] recorded m.p. 213–4°) was boiled with 250ml. of methanol containing 15g. of concentrated sulfuric acid under reflux for two hours and a half. The solution was poured into 500ml. of water, and the separated oil was extracted with petroleum ether. Washing and distillation of the organic layer gave allethrolone-methylether (10g.), b.p. 72–3°/2mm., n_D^{20} 1.4880. By the standard method, the semicarbazone was prepared. m.p. 174–5° (*Anal.* Found: C, 66.59; H, 8.99; N, 15.62. Calcd. for $C_{10}H_{16}ON_2$: C, 66.63; H, 8.95; N, 15.54.).

Attempted synthesis of the methylether by boiling the free allethrolone with methanol and sulfuric acid under the same conditions resulted in a very poor yield (below 4%) of the desired ether.

Ozonolysis and potassium hypobromite oxidation

Allethrolone-methylether (5g.) dissolved in chloroform (30ml.) was treated with excess of ozone at 0°. The solvent was evaporated under reduced pressure and the residual ozonide was decomposed with 5% sodium hydroxide. After being freed from neutral products by extraction with ether, it gave the crude acid product (3g.). This acid was positive against carbonyl reagents. The crude acid (3g.) was dropped into 10% potassium hypobromite solution (150ml.) at 10° with stirring, and stirring was continued at 70° for half an hour. After decomposing the excessive hypobromite by the addition of sodium bisulfite, the reaction mixture was extracted with ether to remove bromoform formed, and the aqueous solution was acidified with dil. sulfuric acid and was continuously extracted with ether by percolation for twenty-four hours.

2) Milton S. Schechter, Nathan Green and F. B. LaForge, *J. Am. Chem. Soc.*, **71**, 3172 (1949).

Evaporation of the ether extract gave α-methoxysuccinic acid, m.p. 101–4°, (1,0 g.). Recrystallizations from ethyl acetate-chloroform gave pure acid in prisms, m.p. 104–5°, not depressed by the admixture of the authentic specimen prepared by Purdie's procedures[3], (*Anal.* Found: C, 40.77; H, 5.58; CH_3O, 20.1. Calcd. for $C_5H_8O_5$: C, 40.54; H, 5.44; CH_3O, 20.9), equivalent weight by titration Found: 73.88 Calcd. For $C_3H_6O\cdot(COOH)_2$ 74.05. By the standard method with diazomethane, the dimethylester was prepared. b.p. 67–9°/5 mm., n_D^{20} 1.4291. For IR-spectra, see the Fig. in the text.

Permanganate and potassium hypobromite oxidations.

Allethrolone-methylether (5 g.), suspended in water (200 ml.), was cooled in ice-water and, to this, 3% permanganate solution (700 ml.) was added dropwise with stirring during four hours, until the permanganate solution was no longer decolourlized. The product was acidified with sulfuric acid and extracted with ether. Evaporation under high vacuum of the ether solution gave keto-acid (VI). Yield 2.3 g. 2,4-Dinitrophenylhydrazone m.p. 105° (*Anal.* Found: C, 44.29; H, 4.47; N, 17.13, Calcd. for $C_{12}H_{14}O_7N_4$: C, 44.17; H, 4.33; N, 17.17). By the same procedures as described above, the keto-acid (2.3 g.) was oxidized with potassium hypobromite (10 w/v%, 70 ml.) and also gave α-methoxysuccinic acid (V), m.p. and mixed m.p. 104–5°, (1.0 g.), together with bromoform.

3) Puride and W. Marshall, *J. Chem. Soc.*, **67**, 946.

Acknowledgement The authors are indebted to Profs. S. Takei and M. Ohno of Kyoto University for their useful advice and also to Mr. K. Uyeyama, President of this Company, for his encouragement in the course of this work.

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Relationship between Stereoisomerism and Biological Activity of Pyrethroids

Part V. The Absolute Configuration of (+)-Pyrethrolone and (+)-Cinerolone*

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Received September 1, 1958

The oxidative degradations of the methyl-ethers of naturally derived pyrethrolone and cinerolone eventually afford (−)-α-methoxysuccinic acid. This chemical conversion without disturbance of the asymmetric centre concerned, establishes, beyond doubt, the absolute configuration of the asymmetric $C_{(4)}$ involved in (+)-pyrethrolone and (+)-cinerolone in the ultimate correlation between these alcohols and (−)-glyceraldehyde *via* the key intermediate, (−)-α-methoxysuccinic acid.

Remarkable advances have been made in the field of pyrethrum chemistry during the last decade and almost all important problems, i.e. structural and synthetic, have successively been dissolved by many workers. However, the sole pending important problem is the elucidation of the absolute configuration of naturally derived pyrethrolone and cinerolone. These two *cyclo*pentenolones constitute the alcohol-moieties of the pyrethrins and their

* Part IV: This Bulletin, **23**, 171 (1959).

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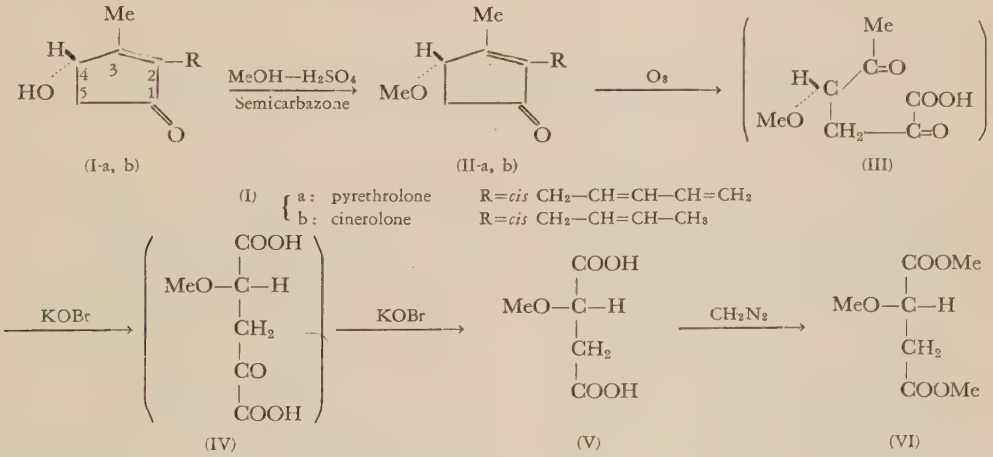
optical activities as well as those of the acid-moieties have been recognized to affect to a greater extent, the insecticidal toxicity of pyrethroids. However, so far little is known in regard of the absolute configuration of the asymmetric $C_{(4)}$ in both (+)-pyrethrolone and (+)-cinerolone, lacking knowledge of which, biological action of the pyrethroids associated with the stereochemical conformation could not be comprehensibly studied.

The extreme ease of racemization has been observed and the fact that both alcohols resemble each other in dextrorotation suggests that they belong to the same stereochemical series. In the present paper, is described the establishment of the chemical correlations of naturally derived pyrethrolone and cinerolone with the standard substance, (−)-glyceraldehyde.

absolute value of optical rotation of active malic acid is too low to permit observations with reliable precision and furthermore, this will be enhanced by the partial racemization during the processes because of the reported extreme ease of racemization of these parent compounds. In contrast with this, an optically active α-methoxysuccinic acid, the methylated form of malic acid, is with optical rotation of a measurable order of magnitude and has also been related to active malic acid by Reichstein[2]. This compounds should also result from the oxidative degradation of pyrethrolone- and cinerolone-methylethers. This was already shown to be feasible by the preliminary work which dealt with the stepwise oxidations of allethrolone-methylether to yield α-methoxysuccinic acid. Pyrethrolone-methylether was at

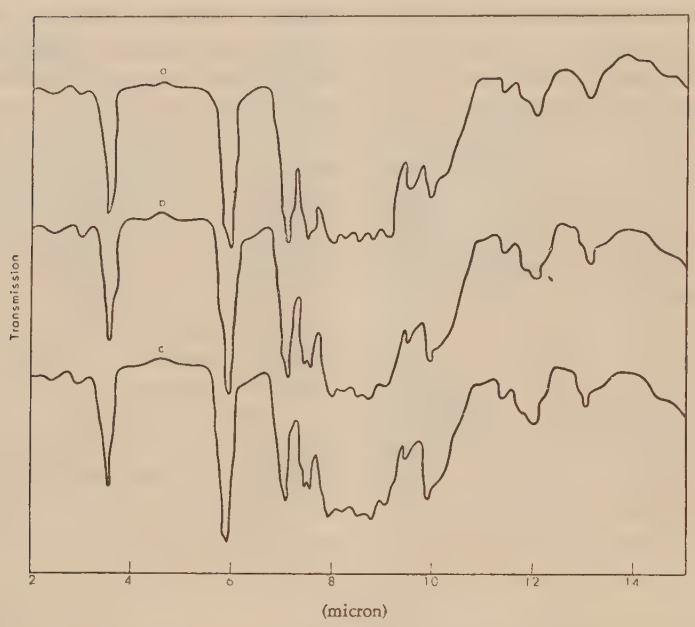


The *cyclo*pentenolone structure of pyrethrolone and cinerolone suggests that, when undergoing a sequel of appropriate oxidations, they should eventually afford an optically active malic acid of either rotation. Malic acid was already related to glyceraldehyde by Freudenberg[1] and, if an optically active malic acid might result from the oxidative degradation, it would provide the ultimate correlation between these alcohols and glyceraldehyde. However, here will be anticipated an experimental difficulty that the first, prepared by Staudinger and Ruzicka[3] and later by LaForge[4] in connection with the elucidation of structure of pyrethrolone, but nothing has been recorded by these authors on the optical activity of this compound.

Consequently, the present authors traced the preparative procedures originated by Staudinger and obtained pyrethrolone-methylether by boiling the naturally derived pyrethrolone-semicar-

1) K. Freudenberg, *Ber.*, **47**, 2027 (1914).

2) A. Lardon and T. Reichstein, *Helv.*, **32**, 2003 (1949).
3) H. Staudinger and L. Ruzicka, *Helv.*, **7**, 212 (1924).
4) F. B. LaForge and H. L. Haller, *J. Am. Chem. Soc.*, **58**, 1777 (1936).



Infra-red spectra of a) synthetic (±)-α-methoxysuccinic acid dimethylester[5] (liquid), b) (−)-α-methoxysuccinic acid dimethylester from pyrethrolone-methylether (liquid), c) (−)-α-methoxysuccinic acid dimethylester from cinerolone-methylether (liquid).

bazone in methanol with a small amount of sulfuric acid. The resulting pure pyrethrolone-methylether had a dextrorotation of $[\alpha]_D^{26}+10.5°$.

Cinerolone-methylether, obtained in exactly the same way, also had a dextrorotation of $[\alpha]_D^{26}+10.0°$. These values are of the order of magnitude, to be expected from those of the parent pyrethrolone and cinerolone, and this indicates the retention of optical activity during the etherification reaction. On the contrary, the etherification of free pyrethrolone under the same reaction conditions resulted in a substantial racemization of the ether only obtained in an inferior yield.

The stepwise oxidation was carried out with the dextrorotatory pyrethrolone- and cinerolone-methylethers, according to the procedures in the preliminary work[5]. Thus, pyrethrolone-methylether (IIa) was at first ozonized and, among the ozonolysis products, neutral fraction, volatile and ether-insoluble acids were removed, leaving a laevorotatory acid which, though not isolated pure, is probably assumed to be the keto-acid (III) from the subsequent reaction. The potassium hypobromite oxidation of the crude keto-acid (III) gave (−)-α-methoxysuccinic acid (V), m.p. 89–91°, $[\alpha]_D^{27}-25.0°$, together with bromoform. The formation of bromoform and the consumption of about 6 mols of hypobromite suggest that this oxidation proceeds through the intermediate α-methoxy-α′-ketoglutaric acid (IV). In a similar manner, (+)-cinerolone-methylether (IIb) also afforded (−)-α-methoxysuccinica cid (IV), m.p. 89–92°, $[\alpha]_D^{27}-24.6°$.

It is recorded in the literature[6] that optically pure laevorotatory α-methoxysuccinic acid melts at 88–9° and has a rotation of −58.18° in acetone. In spite of the slight discrepancies in these physical properties observed with the present authors' samples, which might be ascribed to a partial racemization during the pro-

5) This Bulletin, **23**, 171 (1959).

6) Purdie and W. Marshall, *J. Chem. Soc.*, **67**, 946.

cesses, this identity was evidenced by analyses, equivalent weight as well as by infra-red spectrum indicating the absorptions of its functions.

Not to mention, only α-methoxysuccinic acid can suffice experimental data that the resulting acid is a dicarboxylic acid having the molecular formula $C_5H_8O_5$, containing one methoxy-group and is optically active. Further evidence was provided by complete identity of its methylester (VI) with that of the authentic specimen of the corresponding racemate[5].

Since the *cyclo*pentenolone structures of pyrethrolone, cinerolone and, consequently, of their methylethers have been established and the sole asymmetric carbon atom involved is $C_{(4)}$, it is reasonably concluded that the asymmetric carbon atom in the resulting (−)-methoxysuccinic acid corresponds to that of position-4 in the parent compound, the α-carboxylic carbon to $C_{(3)}$ and the β-carboxylic carbon to $C_{(1)}$ respectively.

The reaction sequence employed here involves no process likely to disturb the asymmetric centre concerned and therefore, the conversions of naturally derived pyrethrolone and cinerolone into (−)-α-methoxysuccinic acid achieve the ultimate correlation between these dextrorotatory alcohols and (−)-glyceraldehyde, thereby establishing the absolute configuration of the $C_{(4)}$-asymmetric centre as I.

EXPERIMENTAL

Melting and boiling points were uncorrected. Microanalyses were carried out by the Microanalytical Division of Mitsui Laboratory of Kyoto University, to whom the authors' thanks are due. Infra-red spectra were recorded with a Parkin-Elmer Double Beam Spectrophotometre (Model 21).

(+)-Pyrethrolone- and (+)-cinerolone-methylethers (+)-Pyrethrolone semicarbazone, prepared by the procedures of LaForge[7], (25 g.), m.p. 212–213°, $[\alpha]_D^{25}$ −155° (*c*, 2.0, glacial acetic acid) in 250 ml. methanol containing 15 g. of concentrated sulfuric acid was boiled under reflux for two and an half hours. The solution was poured into 500 ml. of water and the separated oil was extracted with petroleum ether. Washing and distillation of the organic layer gave (+)-pyrethrolone methylether (9.5 g.), b.p. 95–6°/1.5 mm., $[\alpha]_D^{26}$ +10.5° (*c*, 5.8 ethanol). By the standard method, was prepared the semicarbazone, m.p. 194–5° (methanol-ethylacetate), $[\alpha]_D^{27}$ −40.0° (*c*, 2.0, glacial acetic acid). *Anal.* Found: C, 70.01; H, 8.54; N, 13.77. Calcd. for $C_{12}H_{18}ON_2$: C, 69.87; H, 8.80; N, 13.58).

Attempted preparation of pyrethrolone-methylether by boiling the free pyrethrolone with methanol-sulfuric acid under the same reaction conditions resulted in complete loss of optical activity of the desired ether obtained in trace.

By the same procedures, (+)-cinerolone semicarbazone (20 g.), m.p. 202–4°, $[\alpha]_D^{25}$ −147° (*c*, 2.0, glacial acetic acid), was converted into (+)-cinerolone-methylether (8 g.), b.p.9 1–2°/1.2 mm., $[\alpha]_D^{26}$ +10.0° (*c*, 5.5 ethanol). Semicarbazone m.p. 184–6°, $[\alpha]_D^{27}$ −36.0° (*c*, 2.0, glacial acetic acid). (*Anal.* Found: C, 67.87; H, 9.29; N, 14.49. Calcd. for $C_{11}H_{18}ON_2$: C, 68.00; H, 9.34; N, 14.42).

Ozonolysis and potassium hypobromite oxidation Pyrethrolone-methylether (6 g.) dissolved in chloroform (30 ml.) was treated with excess of ozone at 0°. The solvent was removed under reduced pressure and the residual ozonide was decomposed with 5% sodium hydroxide. The solution was freed from neutral products by extraction with ether and after acidification with dilute sulfuric acid, it was extracted with ether. The ether solution was completely dried in high vacuum at 50° and the residue was again extracted with absolute ether. The removal of ether yielded an acid product with a laevorotation, ca. −5°. Yield 3.2 g..

The crude acid (3.2 g.) was dropped into 10% (w/v) potassium hypobromite solution (150 ml.) at 10° with stirring, and stirring was continued at 70° for half an hour. The excess of hypobromite was decomposed with sodium bisulfite and the separated bromoform was removed by extraction with ether. The aqueous layer was acidified with sulfuric acid and extracted with ether by percolation for twenty-four hours. Evaporation of the extract and the repeated recrystallizations of the residue gave (−)-α-methoxysuccinic acid (1.0 g.), m.p. 89–91°, $[\alpha]_D^{27}$ −25.0° (*c*, 5.0, acetone) (*Anal.* Found: C, 40.73; H, 5.61; CH_3O, 20.3. Calcd. for $C_5H_8O_5$: C, 40.54; H, 5.44; CH_3O, 20.9), equivalent weight by titration Found: 73.80. Calcd. for $C_3H_6O(COOH)_2$: 74.05. Recorded by Purdie: m.p. 88–9°, $[\alpha]_D^1$ −58.18° (*c*, 15.614, acetone)[5]. By applying the standard method with diazomethane, the dimethylester was prepared.

7) F. B. LaForge and W. F. Barthel, *J. Org. Chem.*, **10**, 106 (1945).

b.p. 67–9°/5 mm., n_D^{20} 1.4319, $[\alpha]_D^{27}$ −20.4° (*c*, 3.0, acetone). Recorded by Reichstein: b.p. 181–112°/11 mm., $[\alpha]_D^{16}$ −47.8° (*c*, 3.047, acetone)[2]. The IR-spectrum of the methylester was completely identical with that of the authentic racemic specimen prepared in the preceding paper[5]. (see Fig. in the text.).

By the same procedures, (+)-cinerolone-methylether (5 g.) also afforded (−)-α-methoxysuccinic acid (0.7 g.), m.p. 89–92°, $[\alpha]_D^{27}$ −24.6° (*c*, 5.0, acetone) (*Anal.* Found: C, 40.28; H, 5.62. Calcd. for $C_5H_8O_5$: C, 40.54; H, 5.44), equivalent weight by titration. Found: 73.91. Calcd. for $C_3H_6O(COOH)_2$: 74.05. By the standard method with diazomethane, the methylester was prepared, b.p. 67–9/5 mm., n_D^{20} 1.4301, $[\alpha]_D^{27}$ −19.8° (*c*, 3.0, acetone). The IR-spectrum was also identical with that of the authentic racemate (see the Fig. in the text).

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Studies on Lignin

Part VI. Hydrogen Sulfide Cooking and Subsequent Alkali Treatment of Guaiacylpropanediols and Several Other Lignin Model Compounds

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Coniferyl alcohol, 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1,3) and 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1,2) have been found to react with hydrogen sulfide. From the results of paper chromatography and U.V. spectra a portion of the sulfidation products obtained from the first and second compounds appear to be identical. Upon alkali treatment, these products readily yielded a double bond conjugated to aromatic nucleus. From the last-named compound, a crystalline compound was obtained and its structure is discussed here. In accordance with previous works, it was found that symplocosigenol and dehydrodi-isoeugenol do not react with hydrogen sulfide.

Concerning the reactions of lignin in sulfate process, it is comprehended that at the beginning of cooking, α-position of the phenylpropane side chain is at first bloked with sulfur and later most of sulfur splits off through the action of hot alkali thus leaving carboxyl and carbonyl groups and double bond conjugated to aromatic nucleus. At the same time, hot alkali splits the phenol ethers in lignin so that lignin becomes soluble in alkali[1),2)]. The fact that thiolignin does not show a sharp U.V. spectrum has been explained by assumption of the presence of the above mentioned groups at the α-position[2)]. In Table I, the U.V. spectra of typical lignin model compounds having such

1) T. Enkvist, M. Moilanen and B. Alfredsson, *Svensk Papperstidn.*, **52**, 53 (1949).
2) H. Mikawa, K. Sato, C. Takasaki and K. Ebisawa, *Bull. Chem. Soc. Japan*, **29**, 254 (1956).

TABLE I.

	Type	Compound	U.V. λ_{max} (mμ)	
A	R–C=	propyl guaiacol[3]	227	282
		vanillyl alcohol[3]	230	280
B	R–C=C–C	coniferyl alcohol[4]	267	(297)
		isoeugenol[3]	263	(300)
C	R–CO=	vanillin[5]	278	308
		α-hydroxy propiovanillone[5]	280	303
D	R–COOH	vanillic acid	258	292

R=4-hydroxy-3-methoxyphenyl

groups and those having a saturated carbon atom at the α-position are shown and are classified into four types.

Hydrogen sulfide cooking of lignin model compounds has been investigated by Enkvist, Moilanen[6], Mikawa[7),8)], Zentner[9] and recently by Gierer[10] and alkali treatment of the sulfidation products has also been examined. This paper is concerned with similar experiments on several other lignin model compounds.

The possibility of adding hydrogen sulfide to double bonds of lignin model substances has been investigated by Enkvist, Moilanen[6] and Gierer[10], employing eugenol, isoeugenol and coniferin but only negative results were obtained. The present authors have cooked coniferyl alcohol (I) with a hydrogen sulfide solution of pH 7 for seven hours at 100°C and examined the product applying a paper chromatographic procedure. In addition to a spot of unreacted coniferyl alcohol, a new spot revealing a positive color reaction of sulfide[11] and U.V. spectrum of type A was observed. As shown in Scheme I, U.V. spectrum of this spot was readily converted into type B upon heating with dilute alkali at 100°C for two hours. Thus the splitting of sulfur and simultaneous regeneration of the double bond were proved, though the resulting product was not identical with the starting coniferyl alcohol. In connection with the sulfidation of 1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 3) (II), these products derived from coniferyl alcohol will be discussed later.

(I) R–CH=CH–CH_2OH $\xrightarrow{H_2S}$ Type A $\xrightarrow[100°C]{NaOH}$ Type B

(II) R–CHOH–CH_2–CH_2OH $\xrightarrow{H_2S}$ Type A $\xrightarrow[100°C]{NaOH}$ Type B

(III) R–CHOH–CHOH–CH_3 $\xrightarrow{H_2S}$ Type A $\begin{cases} \text{Crystalline product-probably (VI) or (VII)} \\ \text{water soluble matter-(IV)} \\ \text{resinous matter} \xrightarrow[100°C]{NaOH} \text{Type A} \end{cases}$

SCHEME I

1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 3) (II)[12] was cooked with a hydrogen sulfide solution in the same manner. The reaction product showed U.V. spectrum of type A and gave color reaction of sulfide. From the result of paper chromatography, a portion of the sulfidation product appeared to be identical with that of coniferyl alcohol. Although no definite structure of the reaction product has been established, from the following reason, it appears reasonable to assume that sulfur is bound to α-carbon atom;

3) G. Aulin-Erdtman, *Svensk Papperstidn*, **56**, 91 (1953).
4) K. Freudenberg und G. Schmacher, "Sitzungsber. Heidelberger Akad. Wiss. Math. natur. Klasse", 1956.
5) R. F. Patterson and H. Hibbert, *J. Am. Chem. Soc.*, **65**, 1864 (1943).
6) T. Enkvist and M. Moilanen, *Svensk Papperstidn.*, **55**, 668 (1952).
7) H. Mikawa, *Bull. Chem. Soc. Japan*, **27**, 50 (1954).
8) H. Mikawa, K. Sato, C. Takasaki and K. Ebisawa, *ibid.*, **29**, 265 (1956).
9) T. G. Zentner, *Tappi*, **36**, 517 (1953).
10) J. Gierer and B. Alfredsson, *Acta Chem. Scand.*, **11**, 1516 (1957).
11) H. M. Winegard and G. Toennies, *Science*, **108**, 506 (1948).
12) T. Ishihara and T. Kondo, This Bulletin, **21**, 250 (1957).

Since the addition of SO_3H^- to a double bond of cinnamic aldehyde has been proved to take place at the α-position[13], it seems likely that SH^- is also added at the same position of coniferyl alcohol, and that from the result of experiments on model compounds of *p*-hydroxybenzyl alcohol type[6),7)], benzyl alcohol of 1, 3-diol is also supposed to react with SH^-.

Upon alkali treatment at 100°C for two hours, U. V. spectrum of the sulfidation product of 1, 3-diol readily changed to type B. (Scheme I). Paper chromatographic examination of the product revealed the occurrence of coniferyl alcohol and another unknown spot having U. V. spectrum of type B. This unknown substance also showed the same R_F value as the product obtained from hydrogen sulfide cooking and subsequent alkali treatment of coniferyl alcohol.

The ease with which sulfidation products of coniferyl alcohol and 1, 3-diol convert into type B upon alkali treatment may serve to account for the discrepancy of Zentner's[9)] and Mikawa's[8)] experiments on hydrogen sulfide cooking of 1-(4-hydroxy-3-methoxyphenyl)-propanol-(1) (V) (Table II). Although Zentner claimed of cooking neutral pH, the fact that Zentner obtained isoeugenol, whereas Mikawa identified bis-[1-(4-hydroxy-3-methoxyphenyl)-1-propyl] disulfied and its corresponding monosulfide may be explained from the difference in pH of cooking solutions.

Upon heating with alkali at 160°C, the sulfidation product of 1, 3-diol yielded vanillin and several other unidentified substances.

No proof of the occurrence of the 1, 3-diol structure in lignin is available. However, if the open ring structures of dehydrodi-coniferyl alcohol and pinoresinol which were presented by Freudenberg as intermediate products of biosynthesis of lignin[14)] are to be considered, these should assume 1, 3-diol structure.

1-(4-hydroxy-3-methoxyphenyl)-propanediol-(1, 2) (III)[12)] yielded a crystalline product, resinous matter and water soluble compound, upon cooking with a hydrogen sulfide solution of pH 7 for seventy-two hours at 100°C. As shown in Scheme I, from the aqueous liquor guaiacylacetone (IV) was obtained and was identified as its semicarbazone. This fact is in accordance with the earlier work that the veratryl analogue of this diol gave veratrylacetone upon heating with acid[15)]. That the dehydration of 1, 2-diol yields guaiacylacetone is of interest, since the same guaiacylacetone has also been found in ethanolysis oil of lignin[16)].

Resinous matter having U. V. spectrum of type A did not show any appreciable change in its U. V. spectrum upon heating with dilute alkali at 100°C. However, when this matter was heated to 160°C, some change was observed in U. V. spectrum, but there was no decrease in the sulfur content.

TABLE II.

(IV)	R–CH_2–CO–CH_3	(IX)	R–CH_2OH
(V)	R–CHOH–CH_2–CH_3	(X)	R–CH_2SSCH_2–R
(VI)	R–CH(S)–CH(O)–CH_3 R–CH(S)–CH(O)–CH_3	(XI)	R–CH_2SCH_2–R
		(XII)	R′–CH_2OH
(VII)	R–CH(S)–CH(O)–CH_3 CH_3–CH(S)–CH(O)–R	(XIII)	R′–CH_2SCH_2–R′
		(XIV)	R′–CH_2SSCH_2–R′
(VIII)	CH_2(S)–CH_2(O) CH_2–CH_2	R′ = 4-hydroxy-3,5-dimethoxyphenyl	

The yield of the crystalline product varied on each cooking procedure being 17% in maximum, while in some cases no crystalline compound was obtained. The reason why this crystal is not always obtained has not been elucidated yet. The compound, having U. V. spectrum of type A, gave positive color reactions of phenol and sulfide. Sulfur- and molecular weight-determinations established that the compound was a dimer of phenylpropane con-

13) F. E. Brauns, "The Chemistry of Lignin", Academic Press, New York, 1952, p. 388.
14) K. Freudenberg, "Fortschr. Chem. Org. Natur Stoffe XI", Springer, Wien, 1954 p. 43.
15) E. Adler, *Svensk Papperstidn.*, **55**, 563 (1952).
16) E. West, A. S. MacInnes and H. Hibbert, *J. Am. Chem., Soc.*, **65**, 1187 (1943).

taining one sulfur atom. Acetylation and benzoylation proved the presence of two hydroxyl groups per dimer. Since both hydroxyl groups are supposed to be phenolic, from the result of U. V. spectra determined in neutral and alkaline solutions, four alcoholic hydroxyl groups which are originally present in two mols of diol should have disappeared. Based on these data, either the formula (VI) or (VII) (Table II) might tentatively be proposed for this compound. It is already known that *p*-hydroxybenzyl alcohols react with hydrogen sulfide to form monosulfide or disulfide[6),7)], and that the same alcohols are readily dehydrated to form ethers upon heat treatment[17)]. Such a six membered ring which contains a sulfur and an oxygen has been known as thioxane (VIII)[18)]. There may be some possibility that residual sulfur in thiolignin also exists in such a type. No great significance, however, can be attached to this conception until more complete verifications on the presence of thioxane-sulfur are attained.

As stated above, it has already been established that *p*-hydroxybenzyl alcohols and ethers react with hydrogen sulfide to form monosulfides or disulfides[6),7)]. On the other hand, it has also been reported that pinoresinol and dihydrodehydrodi-isoeugenol do not react with hydrogen sulfide in spite of the presence of *p*-hydroxybenzyl ethers in a form of hydrofuran ring[6),10)]. We have also conducted the hydrogen sulfide cooking of symplocosigenol, a diastereoisomer of pinoresinol[19),20)], and dehydrodi-isoeugenol[21)] and have recovered them. Thus, the stability of benzyl ethers of such a type was verified again.

In a previous communication[22)], a reexamination of hydrogen sulfide cooking of vanillyl alcohol (IX) and the conversion of vanillyl disulfide (X) into vanillyl monosulfide (XI) were briefly reported. In case of hydrogen sulfide cooking of vanillyl alcohol in acetate buffer solutions, vanillyl disulfide was obtained from slightly acidic pH solutions and vanillyl monosulfide from neutral or slightly alkaline solutions. The result of similar experiments on syringyl alcohol (XII), however, showed that slightly acidic solutions yielded only syringyl monosulfide (XIII) or a mixture of syringyl disulfide (XIV) and monosulfide. Although no definite conditions for the disulfide formation have been available, it is certain that the slightly alkaline solution affords monosulfide, since hydrogen sulfide cooking of vanillyl disulfide at pH 9 yielded vanillyl monosulfide. As reported previously, vanillyl disulfide was found to be converted into vanillyl monosulfide accompanied with the formation of vanillin and hydrogen sulfide upon cooking with borate buffer solutions of pH 10 or 11 containing no hydrogen sulfide. In similar cooking, syringyl disulfide was also found to be converted into syringyl monosulfide.

17) B. O. Lindgren, *Svensk Papperstidn.*, **55**, 78 (1952).
18) H. T. Clarke, *J. Chem. Soc.* **1912**, 1806.
19) K. Nishida, M. Sumimoto and T. Kondo, *J. Jap. Forest Soc.*, **33**, 235, 269 (1951).
20) H. Erdtman, "Moderne Methoden d. Pflanzenanalyse III", Springer 1955 p. 439.
21) B. Leopold, *Acta Chem. Scand.*, **4**, 1523 (1950).
22) T. Ishihara and T. Kondo, This Bulletin, **22**, 203 (1958).

EXPERIMENTAL

All cookings were carried out in four 12.5 ml stainless steel tubes. These tubes were place in an autoclave and shaken continuously during cooking.

Coniferyl alcohol (I) Coniferyl alcohol (0.4 g) prepared according to Freudenberg[23)] was cooked for seven hours at 100°C with a Clark-Lubs phosphate buffer solution (50 ml) of pH 7 in which hydrogen sulfide was bubbled through. The solution, after removal of a small amount of insoluble material and the expulsion of hydrogen sulfide under reduced pressure was extracted with ether, and the extract was examined by paper chromatography using water saturated with benzene as a developing agent[14)]. Besides the spot of unreacted coniferyl alcohol (R_F 0.6, violet color with diazotized sulfanillic acid), a new spot (0.75, red) was observed. The latter was also detected as a bleached area against a pink background when it was sprayed with a mixture of 0.066M potassium iodide and 0.0033M chloroplatinic acid. This reagent has been reported by Winegard and Toennies to be able to detect sulfur containing amino acid[11)]. The U.V. spectrum of the ethanol extract of this spot was of type A (λ_{max} 282 mμ),

23) K. Freudenberg und H. H. Hubner, *Chem. Ber.*, **85**, 1181 (1952)

The extract was evaporated to dryness, and the residue was dissolved in 5% sodium hydroxide and heated on a boiling water bath for two hours. The solution was acidified with dilute sulfuric acid and extracted with ether. The extract was dried over sodium sulfate and evaporated to dryness and the residue dissolved in ethanol. The U.V. spectrum of the solution was type B (λ_{max} 266 mμ, hump around 300 mμ). However, the paper chromatogram of the product shows a different R_F value (0.75) and color(red) with coniferyl alcohol.

1,3-diol (II) 1,3-diol (0.7 g)[12] was cooked in a manner similar to that described above. The ether extract of the aqueous solution (0.4 g of colorless oil) was examined by paper chromatography using water saturated with benezene as a developing agent. The paper chromatogram showed two spots, R_F 0.5–0.55 and 0.75; both gave positive color reactions of phenol and sulfur compound. The R_F value, 0.75, was identical with that of the sulfidation product of coniferyl alcohol. The U.V. spectrum of the ethanol extract of this spot was of type A (λ_{max} 281 mμ). After treatment with 5% sodium hydroxide at 100°C for two hours the U.V. spectrum changed to type B (λ_{max} 267 mμ, hump around 300 mμ). R_F value of the product (0.75) was the same as that of the desulfurization product derived from coniferyl alcohol.

The oil obtained from the hydrogen sulfide cooking of 1,3-diol was treated with 5% sodium hydroxide solution at 100°C for two hours. The ether extract of the acidified solution showed an U.V. spectrum of type B (λ_{max} 267 mμ, hump around 300 mμ). Paper chromatogram of the extract showed two spots, 0.6 (violet) and 0.75 (red), of which the former was supposed to be coniferyl alcohol judging from its R_F value and characteristic coloration with diazotized sulfanilic acid.

The oil obtained from the hydrogen sulfide cooking of 1,3-diol was treated with 5% sodium hydroxide solution at 160°C for three hours. The ether extract of the acidified solution was examined by paper chromatography using butanol saturated with 2% aqueous ammonia. Besides the spot vanillin (R_F 0.4), several other unidentified spots were observed. U.V. spectrum of the extract showed λ_{max} 273 mμ and a hump around 300 mμ indicating the presence of an appreciable amount of type B compounds.

1,2-diol (III) 1,2-diol (0.7 g)[12] was heated for seventy-two hours at 100°C with a Clark-Lubs phosphate buffer solution of pH 7 in which hydrogen sulfide was bubbled through. Though in most cases a resinous matter was obtained, formation of crystalline product was observed in some cases of cooking. After filtration of the resinous matter or the crystalline product and expulsion of hydrogen sulfide under reduced pressure, the aqueous solution was extracted continuously with ether. The extract was dried with sodium sulfate and evaporated to give 0.22 g of yellow oil. This was converted into semicarbazone in the usual way. After two recrystallizations from ethanol, the semicarbazone melted at 154.5–155.5°C. Mixed melting point determination with semicarbazone of guaiacylacetone (IV) prepared according to Hibbert[24] showed no depression. *Anal.* Calcd. for $C_{11}H_{15}O_3N_3$: C, 55.68; H, 6.37. Found: C, 55.60; H, 5.99.

The resinous matter obtained from hydrogen sulfide cooking of 1,2-diol showed U.V. spectrum of type A (λ_{max} 283 mμ). *Anal.* Found: C, 56.94; H, 6.15; S, 8.45; Ash, 1.85. After treatment with 5% sodium hydroxide for two hours at 100°C, the resinous matter showed no appreciable change in its U.V. spectrum (λ_{max} 281 mμ). A portion of 0.3 g of resinous matter obtained from the same hydrogen sulfide cooking was heated with 5% sodium hydroxide solution at 160°C for two hours. The solution was acidified with dilute sulfuric acid and evacuated to remove hydrogen sulfide. The resulting tar was filtered off and the filtrate was extracted with ether. Judging from U.V. spectrum (λ_{max} 270, 280 mμ and hump around 310 mμ), the tar (0.15 g) was supposed to be a mixture containing type A and B compounds. *Anal.* Found: C, 59.68; H, 6.24; S, 8.23; Ash, 2.13. Ether extraction of the filtrate gave 50 mg of oil.

The crystalline compound obtained as a mixture with the resinous matter was recrystallized from ethanol. The yields were 10,5,7,17 and 2% respectively in the five cookings, and in most of the other cases only resinous matter was obtained. U.V. spectrum of the crystalline product was of type A (λ_{max} 230, 282 mμ ($\log \varepsilon = 3.8663$)). Acetylation of the crystal (100 mg) with acetic anhydride in pyridine afforded diacetate (120 mg). Benzoylation of this compound (50 mg) with benzoyl chloride in pyridine yielded dibenzoate (50 mg). Results of molecular weight (Rast) and melting point determinations and elementary analysis are listed in Table III.

Although the analytical value of sulfur for the crystalline compound was 1% larger than the calculated value, the value for the diacetate showed a comparatively good agreement with the calculated value.

24) M. Kulka and H. Hibbert, *J. Am. Chem. Soc.*, **65**, 1184 (1943).

TABLE III.

		C	H	S	M.W.	m.p. °C
Crystalline compound	Calcd. for $C_{20}H_{24}O_5S$	63.82	6.43	8.52	376	——
	Found	63.83	6.39	9.57		200
		64.03	6.41	9.66	397	195–199
		63.68	6.51			195–197
Diacetate	Calcd. for $C_{24}H_{28}O_7S$	62.59	6.17	6.96	460.5	——
	Found	62.56	6.03	7.26	479	226–228
Dibenzoate	Calcd. for $C_{34}H_{32}O_7S$	69.85	5.52			——
	Found	69.55	5.38			232–233

Since the crystalline compound showed a 12 mμ shift in U.V. spectrum (λ_{max} 294 mμ ($\log \varepsilon = 4.0444$)) when it was determined in a 0.1N sodium hydroxide solution[3], the two hydroxyl groups proved by acetylation and benzoylation must be phenolic. No carbonyl band (around 1700 cm^{-1}) was observed in the I.R. spectrum. Based on these data, formula (VI) or (VII) was proposed. However, no direct evidence for the presence of the thioxane ring was available. After treatment with 5% sodium hydroxide at 100°C for two hours, the crystalline compound showed no change in its U.V. spectrum (λ_{max} 233, 282 mμ).

Symplocosigenol Symplocosin, a glucoside obtained from *Symplocos lucida*, was hydrolysed with emulsion to give aglucone symplocosigenol[19][20]. m.p. 140.5°C. A quantity of 0.5 g of symplocosigenol was cooked for seven hours at 100°C with a phosphate buffer solution of pH 7 in which hydrogen sulfide was bubbled through. The solution containing some solid was extracted with chloroform. The solvent was evaporated and the residue was recrystallized from ether. m.p. 140–140.5°C. Mixed melting point determination with the starting material showed no depression. Since symplocosigenol is sparingly soluble in water, similar cooking was also carried out in a 50% aqueous ethanol solution. After cooking, ethanol and hydrogen sulfide were removed under reduced pressure, and the resulting precipitate was collected by filtration, 0.4 g. After recrystallization from ether, the product melted at 139–140°C and showed no depression upon admixture with the starting material.

Dehydrodi-isoeugenol A quantity of 0.4 g of dehydrodi-isoeugenol (m.p. 130.5–131°C)[21] was cooked with a hydrogen sulfide solution in a similar manner. After cooking, the precipitate was collected and recrystallized from ethanol, 0.2 g, m.p. 131–132°C. Mixed melting point determination with the starting material showed no depression. Since dehydrodi-isoeugenol is almost insoluble in water, similar cooking was also conducted in ethanol solution. In this case, dehydrodi-isoeugenol was also recovered unchanged.

Hydrogen sulfide cooking of vanillyl alcohol (IX) in acetate buffer solutions The pH of cooking solutions for vanillyl alcohol, syringyl alcohol, vanillyl disulfide and syringyl disulfide was determined by using a glass electrode.

To solutions (50 ml) of $Na_2S \cdot 9H_2O$ (1.7 g), various amounts of glacial acetic acid were added to prepare acetate buffer solutions of pH 5.0–6.4. Vanillyl alcohol (0.4 g)[25] was cooked for seven hours at 100°C with the solution mentioned above. As reported previously[22], the products obtained from the solutions of pH 5.0–5.7, after one recrystallization from toluene, melted at 126–127°C and showed no depression upon admixture with vanillyl disulfide (X) synthesized according to Manchot and Zahn[26]. *Anal.* Calcd. for $C_{16}H_{18}O_4S_2$: C, 56.80; H, 5.36. Found: C, 56.41; H, 5.20. On the other hand, the products obtained from the solutions of pH 6.3–6.4 showed a melting point of 83–84°C which agreed with vanillyl monosulfide (XI) as reported by Mikawa[7]. *Anal.* Calcd. for $C_{16}H_{18}O_4S$: C, 62.74; H, 5.92. Found: C, 63.08; H, 5.74.

In an attempt to expel oxygen from the cooking solution, hydrogen sulfide was bubbled through the acetate buffer solution prepared as mentioned above (pH 5.0). Cooking of vanillyl alcohol with this solution yielded vanillyl disulfide, which was identified by mixed melting point determination.

Oxidation of vanillyl monosulfide (XI) To vanillyl monosulfide (110 mg) dissolved in glacial acetic acid (4 ml), was added 30% hydrogen peroxide (0.3 ml). After standing overnight, the solution was poured into water. The resulting precipitate (60 mg) was recrystallized from ethanol. m.p. 178–179°C. Although it was identical to the melting point of vanillyl sulfoxide

25) W. H. Carothers and R. Adams, *J. Am. Chem. Soc.*, **46**, 1675 (1924).

26) W. Manchot and C. Zahn, *Ann.*, **345**, 320 (1906).

reported by Mikawa[7], analysis did not agree with the calculated value for vanillyl sulfoxide. *Anal.* Calcd. for vanillyl sulfoxide $C_{16}H_{18}O_5S$: C, 59.62; H, 5.63; S, 9.93. Calcd. for vanillyl sulfone $C_{16}H_{18}O_6S$: C, 56.80; H, 5.36; S, 9.46. Found: C, 56.91; H, 5.20; S, 10.23.

Hydrogen sulfide cooking of vanillyl alcohol (IX) in phosphate buffer solutions A portion of 0.4 g of vanillyl alcohol was cooked for seven hours at 100°C with phosphate buffer solutions in which hydrogen sulfide was bubbled through. The reaction product was recrystallized from toluene. From the solutions of pH 7.6 and 5.8 only vanillyl monosulfide was obtained. From another cooking with a solution of pH 7.0, vanillyl disulfide and vanillyl monosulfide were obtained; when the mixture was recrystallized from toluene, vanillyl monosulfide first crystallized and a small amount of vanillyl disulfide was obtained from the filtrate. These substances were identified by mixed melting point determinations with authentic specimens. In order to prepare a cooking solution of acidic pH, hydrogen sulfide was bubbled through a phosphate buffer solution of pH 7 and then to this solution acetic acid was added to adjust the pH to 5.5. Cooking of vanillyl alcohol with this solution afforded vanillyl monosulfide.

Hydrogen sulfide cooking of syringyl alcohol (XII) in acetate buffer solutions Syringyl alcohol was prepared by catalytic reduction of syringaldehyde according to the method of Carothers and Adams[25]. After recrystallization from hot benzene, syringyl alcohol melted at 131–132°C. Richtzenhain[27] 136°C. *Anal.* Calcd. for $C_9H_{12}O_4$: C, 58.69; H, 6.57. Found: C, 58.60; H, 6.38.

Cooking solutions were prepared from sodium sulfide and acetic acid in a manner similar to that described above. A portion of 0.4 g of syringyl alcohol was cooked for seven hours at 100°C. Result of the cookings was summarized in Table IV.

The product obtained as a resinous matter crystallized by scratching the side of the tube. After recrystallization from toluene, the product obtained from Nos. 3, 4, 5, 6, 8 and 9 melted at 78–82°C. Even after the repeated recrystallization from toluene, the product did not show a sharp melting point. After drying on phosphorus pentoxide at 100°C *in vacuo*, the crystal melted at 81–82°C. Analysis agreed with the value calculated for syringyl monosulfide (XIII). *Anal.* Calcd. for $C_{18}H_{22}O_6S$: C, 59.01; H, 6.05. Found: C, 59.10; H, 5.99.

After standing for two days in open air, syringyl monosulfide showed a broad melting point and elementary analysis agreed with the hydrate. *Anal.* Calcd. for $C_{18}H_{22}O_6S \cdot 2H_2O$: C, 53.72; H, 6.51. Found: C, 53.89; H, 6.56. The water of crystallization was not completely removed by drying over phosphorus pentoxide *in vacuo* at room temperature.

TABLE IV.

No.	pH		Yield (g)	Product
	prior to cooking	after cooking		
1	5.5	5.6	—	not crystallized
2	5.5	6.1	—	D.S. and M.S.
3	5.5	6.15	—	M.S.
4	5.8	6.1	0.31	M.S.
5	6.0	7.0	0.29	M.S.
6	6.15	7.4	0.39	M.S.
7	6.5	7.5	0.35	D.S. and M.S.
8	8.1	10.4	0.3	M.S.
9	9.1	9.4	0.22	M.S.

Syringyl monosulfide (170 mg) was acetylated with acetic anhydride in pyridine. The resulting diacetate (190 mg) was recrystallized from ethanol. m.p. 137–138°C. It was identical with the melting point of syringyl monosulfide diacetate reported by Gierer[10]. *Anal.* Calcd. for $C_{22}H_{26}O_8S$: C, 58.66; H, 5.82. Found: C, 58.40; H, 5.73.

The product obtained from No. 7 (0.35 g) was dissolved in acetone, and the solution was passed through a short alumina column. The solvent was evaporated to dryness and the residue was recrystallized from toluene. The resulting two kinds of crystals were separated by a pincette and recrystallized again. One crystal (150 mg), after acetylation, was identified as syringyl monosulfide diacetate. m.p. 137–138°C. The other (80 mg) showed a m.p. of 123–124.5°C and was identified as syringyl disulfide (XIV) by elementary analysis. *Anal.*

27) H. Richtzenhain, *Chem. Ber.*, **77**, 414 (1944).

Calcd. for $C_{18}H_{22}O_6S_2$: C, 54.27; H, 5.57; S, 16.06. Found: C, 54.34; H, 5.87; S, 16.14.

Hydrogen sulfide cooking of vanillyl disulfide (X) at pH 9 Vanillyl disulfide (0.4 g) was cooked for seven hours at 100°C with an acetate buffer solution of pH 9 prepared from sodium sulfide and acetic acid. The product (0.28 g) was recrystallized from toluene. m.p. 83–84°C. Mixed melting point determination with an authentic vanillyl monosulfide (XI) showed no depression. *Anal.* Calcd. for $C_{16}H_{18}O_4S$: C, 62.74; H, 5.92. Found: C, 62.85; H, 5.72.

Cooking of vanillyl disulfide (X) with weak alkaline solutions Vanillyl disulfide (0.4 g) was cooked for seven hours at 100°C with a borate buffer solution of pH 9.3 (0.2M boric acid in 0.2M potassium chloride 35 ml and 0.2N sodium hydroxide 14.9 ml). The precipitate (0.34 g) was filtered off and recrystallized from ethanol. m.p. 128–129°C. Mixed melting point determination with the starting material showed no depression.

Cooking of vanillyl disulfide (0.4 g) with borate buffer solutions of pH 10 or 11 yielded vanillyl monosulfide, vanillin and hydrogen sulfide. Table V presents the result of determinations of the products and the calculated value from the equation:[22]

$$2R\text{–}CH_2SSCH_2\text{–}R + 2H_2O = R\text{–}CH_2SCH_2\text{–}R + 2R\text{–}CHO + 3H_2S$$

The insoluble material was filtered off, and the filtrate was collected in a 200 ml volumetric flask. The solution was diluted up to the mark and mixed. An exact quantity of 20 ml of the solution was acidified with dilute sulfuric acid, and the hydrogen sulfide was distilled with carbon dioxide into a receiver containing 0.1N iodine solution. Hydrogen sulfide was determined by back titration with 0.1N sodium thiosulfate solution. To another 20 ml of the solution was added 20 ml of 0.5% 2,4-dinitrophenylhydrazine in 3N hydrogen chloride solution, and the resulting vanillin hydrazone was collected on a glass filter, dried at 100°C and weighed.

The yield of vanillyl monosulfide given in Table V indicates the total amount of insoluble material, and there may be possibility that the unreacted vanillyl disulfide is contained. However, after one recrystallization from toluene the product showed a m.p. of 83–84°C and was identified as vanillyl monosulfide by mixed melting point determination with an authentic specimen.

Cooking of syringyl disulfide (XIV) with a weak alkaline solution Syringyl disulfide (20 mg) was cooked for seven hours at 100°C with a borate buffer solution (12.5 ml) of pH 10.0. After cooking, the solution was acidified and the resulting precipitate was collected and examined by paper chromatography using 15% acetic acid as a developing agent. A spot of syringyl monosulfide (R_F 0.67) was detected by spraying diazotized sulfanilic acid or a mixture of chloroplatinic acid and potassium iodide solution[11]. By a similar paper chromatography, the starting material did not afford such a distinct spot as syringyl monosulfide, but showed a tailing from the origin. The filtrate obtained after the removal of syringyl monosulfide was extracted with ether, and the extract examined by paper chromatography using butanol saturated with 2% aqueous ammonia as a developing agent. The spot of syringaldehyde (R_F 0.35) showed a violet fluorescence under ultraviolet light and gave an orange color with 2,4-dinitrophenylhydrazine solution.

TABLE V.

	Vanillyl monosulfide (mg)	Vanillin (mg)	Hydrogen sulfide (mg)	pH: Prior to cooking	pH: After cooking	Recovery of aromatic nucleus (%)
Calcd.	180	180	60			
No. 1	190	90	27	10.1	9.4	77
2	220	83	34	10.0	9.3	83
3	190	88	37	10.0	9.2	77
4	220	117	34	11.0	9.3	93

Acknowledgments The authors wish to express their thanks to Mr. Sato of Kokusaku Pulp Ind. Co. Ltd. for his kind gift of dehydrodiisoeugenol and to Mr. Tanaka and Miss Furusawa of this laboratory for their kindness in supplying the syringaldehyde used here and for performing the microanalysis.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 186~194, 1959]

Studies on the Sunlight Flavour of Beer

Part VI. Correlation of the Occurrence of the Sunlight Flavour of Beer to Humulone, Lupulone and related Compounds

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In order to establish the mechanism of occurrence of sunlight flavour of beer in continuation of the preceding paper, humulone, lupulone, and their analogues and degradation compounds, i.e., cohumulone, adhumulone, 5-acetyl-3-methylfilicinic acid including analogues, tetrahydrohumulone, hexahydrolupulone, humulinic acid and lupuloxinic acid, were tested for the occurrence of sunlight flavour of beer.

As a result, among the above-mentioned compounds, humulone, lupulone, cohumulone, adhumulone, tetrahydrohumulone and hexahydrolupulone were found to cause the sunlight flavour, but the other compounds did not cause typical sunlight flavour. This fact shows that some specific structural components seem requisite for the occurrence of sunlight flavour of beer.

It was also revealed that isomerization caused by boiling accelerates the occurrence of the sunlight flavour of beer. Finally, the result of the experiment conducted by the gas chromatographic procedure showed that any new component is not detected by the exposure of beer to sunlight but, two components somewhat increased.

In part V[1] of this series, the conditions for the occurrence of the sunlight flavour of beer were established.

Among these conditions, the relation between humulone, lupulone, their analogues, degradation products and the occurrence of sunlight flavour was investigated, and the following results were obtained:

(1) In order to clarify the relation of occurrence of sunlight flavour of bear and the following compounds, i.e., cohumulone and adhumulone which are analogues of humulone, these compounds were isolated by the counter current distribution procedure.

As regards the isolation of cohumulone and adhumulone through the counter current distribution procedure, several procedures have been described[2].

As to the distribution solvent, 2,2,4-trimethylpentane has usually been used for the upper phase solvent. Because this solvent is expensive, ligroin (b.p. 90–100°) was used in our work and was found to be suitable for the separation of humulone analogues.

The solvent, as described in the literature, which consisted of 7 volumes of 1M-K_2HPO_4 adjusted to pH 7.3, with concentrated H_3PO_4 and of 3-volumes of methnol was used for the lower phase solvent.

Cohumulone and adhumulone thus obtained were added to wort or to 10% sucrose solution and the mixture was boiled for one hour.

To this was added beer yeast and main fermentation was completed. The mixture was exposed to sunlight for four hours, and then an organoleptic test was carried out.

As a result, both cohumulone and adhumulone were found to be the cause of the flavour as well in the case of humulone, and, when they were boiled with wort or with 10% sucrose

1) Y. Obata and H. Horitsu, This Bulletin, **22**, 153 (1958).
2) F. L. Rigby and J. L. Bethune, *J. Am. Chem. Soc.*, **77**, 2828 (1955).

solution, a stronger flavour more intensely than in the case without boiling occurred.

This fact indicates that isomerization caused by boiling with wort or with 10% sucrose solution accerates the occurrence of sunlight flavour of beer.

There are differences among various species of hops in easiness of the occurrence of sunlight flavour in beer, so comparision test for three components contents, i.e., humulone, cohumulone and adhumulone between Japanese and German species, was carried out by the counter current distribution procedure using ligroin (b. p. 90–100°) and phosphate buffer (pH 7.3).

It was found that the German species contained more cohumulone than the Japanese species.

(2) In order to know the part of humulone or lupulone related to flavour, in other words, which nucleous or side chain part has relation, humulone and lupulone were both degraded in an alkaline medium, and humulinic acid was isolated from humulone, but lupuloxinic acid was not isolated from lupulone.

This means that in alkaline medium lupulone is stable and undergoes no degradation.

Lupuloxinic acid was obtained in the degradation using hydrogen peroxide and alkaline medium. Humulinic acid and lupuloxinic acid thus obtained were both added to the wort or to a 10% sucrose solution to which beer yeast was added and main fermentation was completed.

After being exposed to sunlight for four hours, an organoleptic test was carried out.

In both cases, the occurrence of a typical sunlight flavour could not be observed.

(3) For the purpose of investigating the degradation of humulone and lupulone under conditions similar to beer brewing, humulone and lupulone were boiled, respectively for one hour in phosphate buffer composed of N/5 Na_2HPO_4 and N/5 KH_2PO_4, adjusted to pH 5.5.

It was found that humulone and lupulone were both isomerized to isohumulone and isolupulone and humulone was more extensively isomerized and soluble than lupulone.

This fact indicates that humulone is more significant than lupulone in respect of the occurrence of flavour.

When 10% sucrose solution was added to the phosphate buffer, the same result was obtained.

(4) For the purpose of investigating the relation between the hydrolytic products and the occurrence of flavour, either humulone or lupulone was hydrogenolized with platinum-black and hydrogen in a methanolic medium.

Tetrahydrohumulone and hexahydrolupulone thus obtained, were added to the wort or to 10% sucrose solution and the mixture was boiled for four hours, an organoleptic test was carried out.

It was found that the test was positive in both cases. This fact indicates that as regards form of side chain, the saturated side chain also gives the same result as the unsaturated side chain, namely, that flavour occurs in both saturated and unsaturated side chains.

(5) An experiment was carried out to find the relation between the boiling period and the isomerization of humulone into isohumulone.

Humulone was dissolved in a 0.0135 N methanolic solution of sodium hydroxide and was refluxed.

Exchangeable ration from humulone to isohumulone was spectrophotometrically estimated due to the isohumulone having a maximum absorption peak at 255 mμ, instead of humulone having a maximum at 325 mμ.

Sixty minutes boiling was found sufficient for the isomerization of humulone into isohumulone.

(6) 5-Acetyl-3-methylfilicinic acid and its analogues which have a same nucleous as lupulone, but have a simpler side chain than that of lupulone, i.e., the methyl group, were synthesized according to Riedl's method[3].

This synthesis was carried out using phloroglucinol as the starting material, proceding via phloroacetophenone as the intermediate and 5-acetyl-3-methylfilicinic acid and its analogues were prepared. Among the above-mentioned

3) W. Riedl and K. H. Risse, *Ann.*, **585**, 209 (1954).

compounds, phloroglucinol, phloroacetophenone and 5-acetyl-3-methylfilicinic acid were boiled with a phosphate buffer of pH 5.5, for one hour.

It was found that in phloroacetophenone and 5-acetyl-3-methylfilicinic acid, solubility for phosphate buffer of pH 5.5 was increased with boiling, but in phloroglucinol, solubility was almost unaffected.

These three compounds were added to wort or to 10% sucrose solution, and after beer yeast was added to the mixture, main fermentation completed.

After being exposed to sunlight for four hours, an organoleptic test was carried out.

In the above cases, occurrence of typical sunlight flavour was not observed.

(7) An experiment with application of the gas chromatographic procedure revealed that by the exposure of beer to sunlight, new components are not detectable, but two components somewhat increases.

EXPERIMENT AND RESULTS

(1) Isolation of humulone, cohumulone and adhumulone.

Hops (the Japanese species produced in 1956 and 1957 and the German species produced in 1956) (100 g) were extracted with ether (1 l), and the ethereal solution was extracted with 2N-NaOH (1 l), the 2N-NaOH soluble fraction acidified with dilute sulfuric acid and extracted with ether.

The ethereal solution was then evaporated and the residue extracted with methanol, and methanolic lead acetate solution added to the methanolic soluble fraction to precipitate the lead complex of humulones.

Subsequently, the precipitate was decomposed with dilute sulfuric acid, and free humulones were obtained.

The resulted humulones (500 mg) were distributed into 70-tubes, 10 ml. per phase, counter current apparatus, using ligroin (b.p. 90–100°) as the upper phase solvent and a buffer composed of 7 volumes of 1M-K_2HPO_4 adjusted to pH 7.3 with concd. H_3PO_4 and 3 volumes of methanol was used as the lower phase solvent.

Thence, aliquots of the upper layer phase were taken out and were submitted for the estimation by ultraviolet absorption spectrum at 332 mμ.

The results of Japanese and German species are shown in Fig. 1 and Fig. 2, respectively.

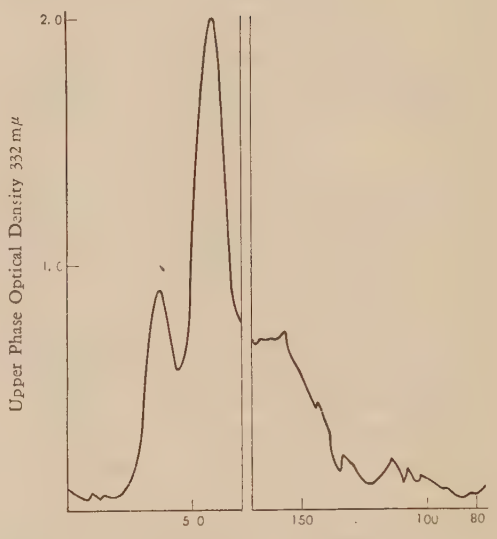


FIG. 1. Distribution Pattern for the Pb-pptd. Fraction of Japanese Hop.

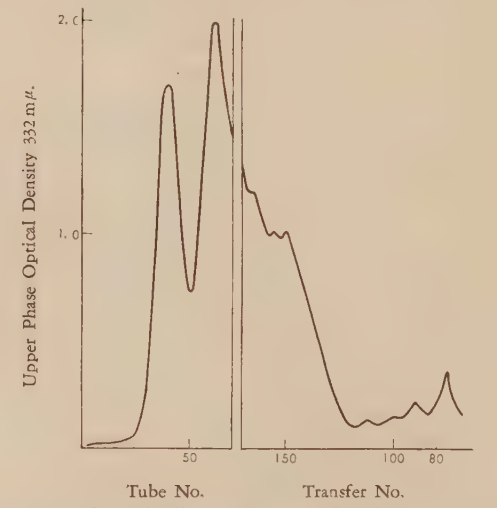


FIG. 2. Distribution Pattern for the Pb-pptd. Fraction of German Hop.

As shown in Fig. 1 and Fig. 2, the German species contain much more cohumulone than the Japanese species does.

The more rapidly moving material, which was principally adhumulone, was recycled. Fig. 3 and Fig. 4 show the result of separation, respectively.

The center portions of humulone, cohumulone, and

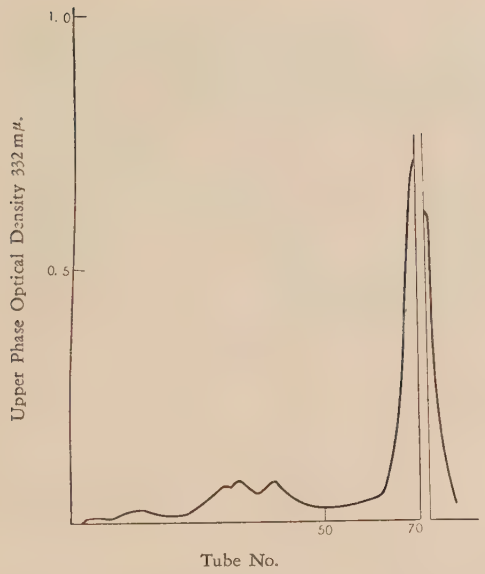


FIG. 3. Redistribution Pattern for the Pb-pptd. fraction of Japanese Hop.

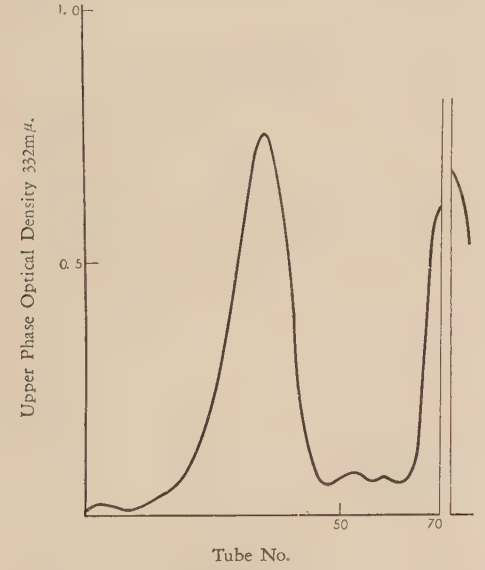


Fig. 4. Redistribution Pattern for the Pb-pptd. Fraction of German Hop.

adhumulone were removed respectively, and the phase were separated. Each component was removed from the ligroin phase by extraction with two successive 100 ml.-portions of 90% methanol.

Humulone, cohumulone and adhumulone thus obtained were added at a concentration of 250 mg/l to wort or to the 10% sucrose solution, and the mixture was boiled for one hour.

To this mixture was added beer yeast and main fermentation completed. After being exposed to sunlight for four hours, an organoleptic test was carried out.

It was found that humulone, cohumulone and adhumulone all contribute to flavour.

(2) Isomerization of humulone and lupulone under similar conditions to beer brewing.

Separation of both humulone and lupulone was carried out by the same procedure as described in the preceding paper (Part V).

Humulone (0.510 mg) or lupulone (0.825 mg) was dissolved in 2 ml. of phosphate buffer composed of N/5 KH_2PO_4 and N/5 Na_2HPO_4, adjusted to pH. 5.5, and the solution was refluxed for one hour, and aliquot was then taken off and diluted with alkaline methanol and was estimated for the UV-absorption spectrum, being compared with the initial UV-spectrum, respectively. The results thus obtained are given in Fig. 5 and Fig. 6, respectively.

As shown in these figures, it is obvious that humulone is much more exchangeable in character than lupulone.

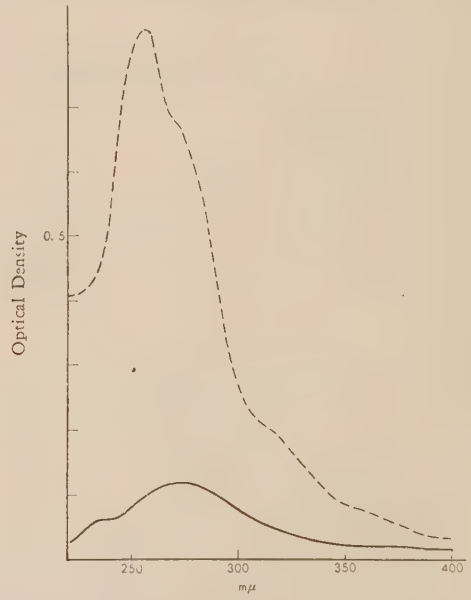


FIG. 5. Absorption Spectra of Exchanged Humulone Refluxed in Phosphate Buffer pH. 5.5.
——, initial. - - -, final

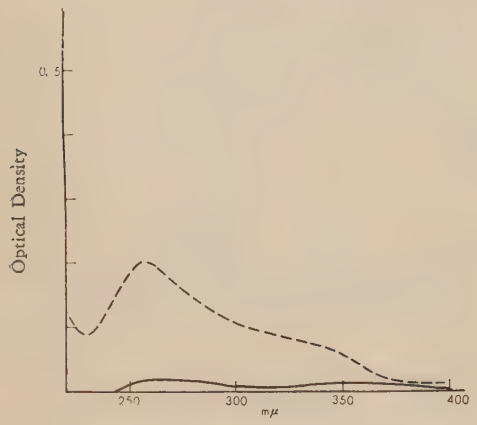


FIG. 6. Absorption Spectra of Exchanged Lupulone Refluxed in Phosphate Buffer pH. 5.5.
——, initial, - - -, final.

Even when the 10% sucrose solution was added to the phosphate buffer containing humulone or lupulone, the resultant curves were identical.

(3) Degradation of humulone and lupulone in the alkaline medium[4,5]

Humulone (100 mg) or lupulone (100 mg) was dissolved in methanol (0.5 ml), and 1N-NaOH (2 ml) added to the solution.

The solution was refluxed for one hour and a half, acidified with dil. HCl, and extracted with ether; the ethereal solution thus obtained was then extracted with dil. $NaHCO_3$ solution.

The $NaHCO_3$-soluble fraction was acidified and extracted with ether.

The ethereal solution was washed with H_2O, dried with anhyd. Na_2SO_4, distilled, and a crude crystalline substance was obtained.

This crystalline was recrystallized from aqueous methanol.

In this process, humulinic acid was obtained but lupuloxinic acid was not obtainable. This fact shows that humulone degrades in alkaline medium to humulinic acid, while lupulone does not degrade to the lupuloxinic acid in the same medium.

Humulinic acid gave m.p. 93°, and the UV-spectrum is shown in Fig. 7.

To obtain lupuloxinic acid, the following experiment was carried out. Lupulone was degraded under hydrogen-peroxide and alkaline medium to lupuloxinic acid.

Lupulone (1.0 g) was dissolved in 10% NaOH (50 ml) solution, and 38% H_2O_2 (10 ml) was added to the solution. After standing for five hours at room temperature, the reactant was poured into ice water, acidified with sulfuric acid, and cooled in the refrigator over night.

Thus the crystalline substance was obtained by the same process as that for obtaining humulinic acid from humulone.

The obtained crystalline substance was lupuloxinic acid and gave m.p. 107°.

The UV-absorption spectrum is shown in Fig. 8.

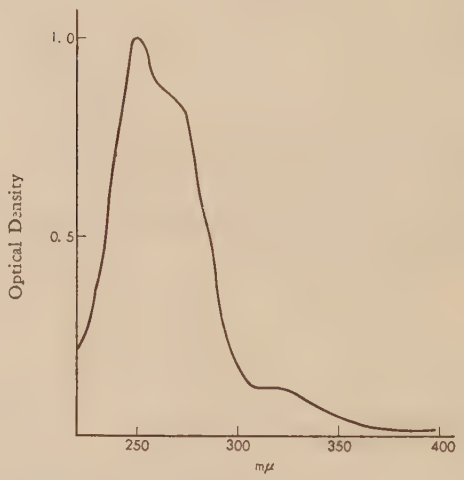


FIG. 7. Absorption Spectrum of Humulinic Acid.
Concentration of 0.315 mg/10 ml. in Alkaline Methanol Solution.

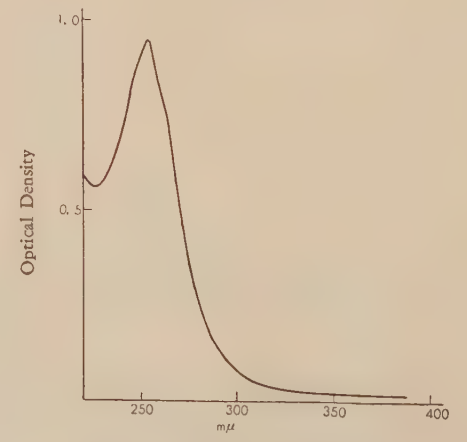


FIG. 8. Absorption Spectrum of Lupuloxinic Acid.
Concentration of 0.75 mg/10 ml. in Alkaline Methanol Solution.

4) G. Harris, *J. Chem. Soc.*, **1952**. 1906.
5) G. A. Howard and J. R. A. Pollock, *J. Chem. Soc.*, **1952** 1902.

Lupuloxinic acid as well as humulinic acid show a maximum absorption peak at 255 mμ.

Humulinic acid (50 mg) or lupuloxinic acid (50 mg) was added to wort (200 ml) to which beer yeast was added and main fermentation completed, after being exposed to sunlight for four hours.

An organoleptic test was carried out but this did not result in typical sunlight flavour.

(4) Hydrogenolysis of both humulone and lupulone[6]

a. Humulone (850 mg) was dissolved in methanol (40 ml), and platinum black (600 mg) was added to the solution and the solution reduced.

After the calculated quantity of hydrogen was absorbed, the reactant filtered, and the filtrate concentrated in vacuo and tetrahydrohumulone was collected. The UV-absorption spectrum is shown in Fig. 9.

This curve shows insignificant difference from that of humulone.

b. Lupulone (720 mg) was dissolved in methanol (40 ml), and platinum black (250 mg) was added to the solution and reduced in the same way as in the case of humulone.

Hexahydrolupulone was thus obtained and UV-absorption spectrum is shown in Fig. 10.

This curve shows insignificant difference from that of lupulone.

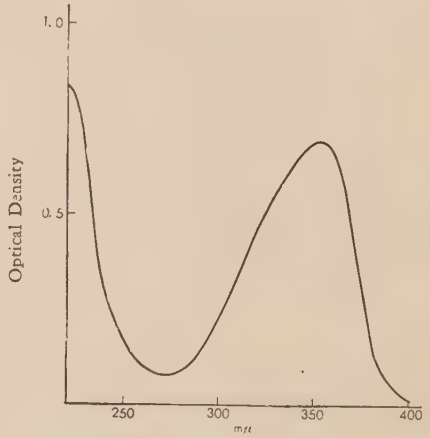


FIG. 10. Absorption Spectrum of Hexahydrolupulone.
Concentration of 0.148 mg/10 ml. in Alkaline Methanol Solution

(5) Isomerization period from humulone to isohumulone.

Humulone (665 mg) was dissolved in 95% ethanol (25 ml) and the mixture giving an excess alkaline concentration of 0.135 N.

After refluxing on a water bath, and sampling (0.1 ml) were carried out in an intervals of several minutes and then the sample taken was diluted to 100 ml. with

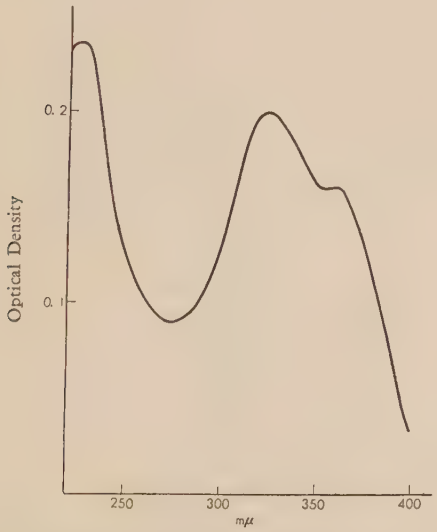


FIG. 9. Absorption Spectrum of Tetrahydrohumulone.
Concentration of 0.102 mg/10 ml. in Alkaline Methanol Solution.

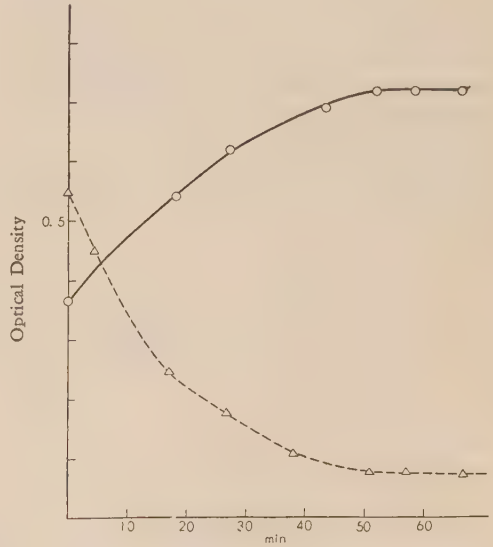


FIG. 11. Isomerization Curves of Humulone to Isohumulone.
○—○, 255 mμ; △---△, 325 mμ

6) J. F. Carson, *J. Am. Chem. Soc.*, **73**, 1850 (1951).

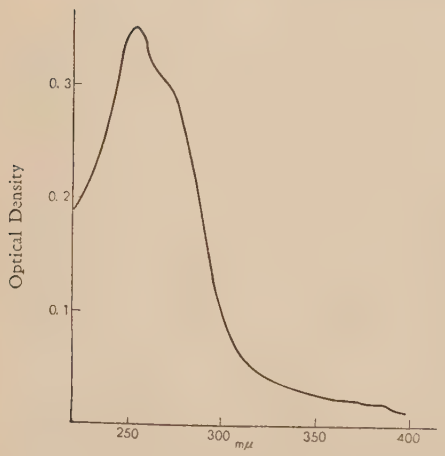


FIG. 12. Absorption Spectrum of Isohumulone.
Concentration of 0.152 mg/10 ml. Alkaline Methanol Solution

methanol; then UV-absorption spectra were estimated at both 325 mμ and 255 mμ, and reaction was stopped when the maximum value at 255 mμ and the minimum value at 325 mμ were obtained.

The period for the isomerization from humulone to isohumulone is shown in Fig. 11, and UV-absorption spectrum of isohumulone is shown in Fig. 12.

As shown in Fig. 11, it is apparent that sixty minutes is sufficient for the isomerization period from humulone to isohumulone.

(6) Synthesis of 5-acetyl-3-methylfilicinic acid (I), 1-acetyl-3,3,5,5,-tetramethyl-cyclohexene-*1*-ol-2-dion (4,6) (II), 3-acetyl-filicinic acid (III), and 3-methyl-phloroacetophenone (IV).

a. Synthesis of Phloroacetophenone.

Phloroglucinol dried at 120° (15 g) was suspended in CS_2 (60 ml), and powdered $AlCl_3$ (48 g) added to the suspension.

Nitrobenzene (45 ml) was dropped for thirty min.; fuming HCl was thus broken out and the reactant was dissolved into the solution. The solution was then warmed to the boiling point of CS_2.

After this, acetylchloride (12 g) dissolved in CS_2 (5 g) was dropped. Thirty minutes later, the reactant was cooled to 30°, poured into ice water (500 ml) containing conc. HCl (5 ml), and instantly, the solvent was evaporated from the reactant, the remaining resin was added into a large amount of boiling water, and allowed to stand, to yield crystalline.

This crystalline gave m.p. 70°; yield 25 g.

The UV-absorption spectrum is shown in Fig. 13.

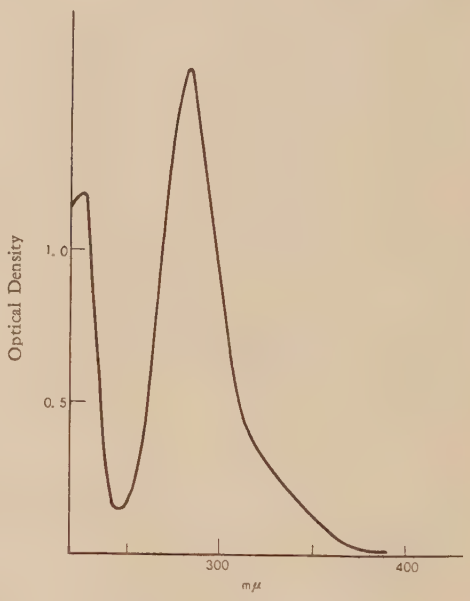
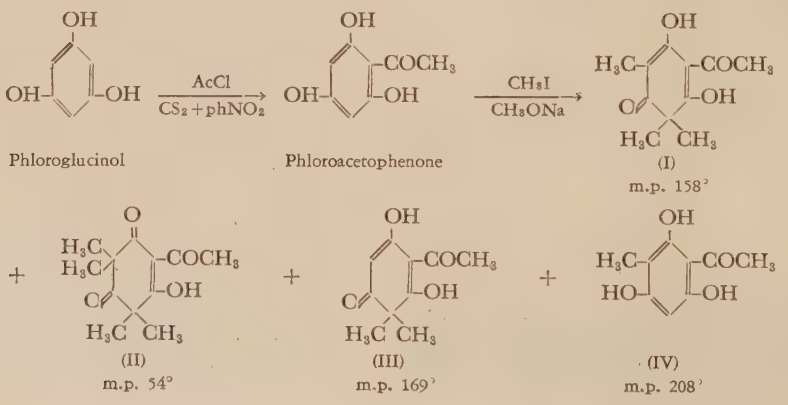


FIG. 13. Absorption Spectrum of Phloroacetophenone.
Concentration of 0.05 mg/10 ml. in Methanol.



b. Synthesis of 5-Acetyl-3-methylfilicinic acid (I), 1-acetyl-3,3,5,5,-tetramethyl-cyclohexene-1-ol-2-dion (4, 6), (II), 3-acetyl-filicinic acid (III), and 3-methylphloroacetophenone (IV).

Dried phloroacetophenone (5 g) was dissolved in absolute MeOH (37.5 ml), and after cooling, Na (2.052 g) dissolved in MeOH (27 ml), was added to the solution followed by the addition of CH_3I (25 g). After addition, the reactant was kept in a desiccator for five days. After this period, the solvent was evaporated at 40° in vacuo under N_2-gas. The remaining syrup was poured into a mixture of H_2O (62.5 ml) and ether (300 ml), and acidified with 2N-H_2SO_4. The 2N-H_2SO_4-soluble fraction was extracted with ether, the ethereal solution extracted with 5% $NaHCO_3$, and the 5% $NaHCO_3$-soluble fraction was acidified with 2N-H_2SO_4 and crystalline substance was collected.

This crystalline substance was digested by cooling benzene and (II), m.p. 54°, was gained after evaporation of the solvent from the benzene-soluble fraction. The benzene-insoluble fraction was dissolved in warm 30% MeOH, and after filtration, (I), m.p. 158°, was crystallized from the filtrate.

The filtrate removing (I), was then diluted with H_2O, and (III), m.p. 169°, was obtained.

Finally, the ethereal solution which removed the 5% $NaHCO_3$-soluble-fraction, was extracted with 10% Na_2CO_3 and the 10% Na_2CO_3-soluble-fraction was acidified with dil. H_2SO_4 to yield crystalline from the solution.

This crystalline (IV), gave m.p. 175°.

The UV-absorption spectrum of (II) is Fig. 14.

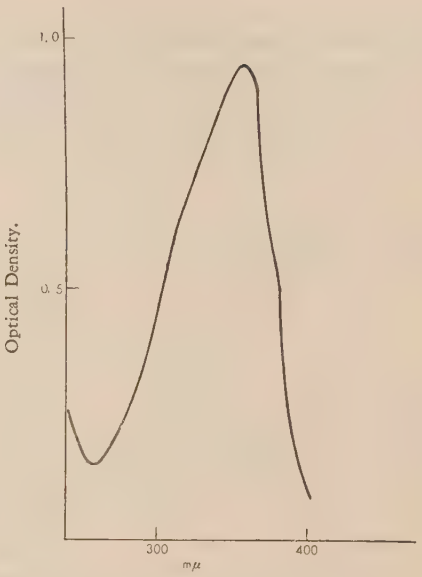


FIG. 14. Absorption Spectrum of 5-Acetyl-3-methylfilicinic Acid.
Concentration of 0.121 mg/10 ml. Ethanol.

Three kinds of beer containing 50 mg of each of phloroglucinol, phloroacetophenone and 5-acetyl-3-methylfilicinic acid per 200 ml of beer, were brewed and after being exposed to sunlight, an organoleptic test was carried out, but no typical sunlight flavour was observed to occur in the three cases.

(7) Analysis of beer by gas partition chromatography.

The partition columns consisted of tricresyl phosphate adsorbed on fire brick.

Beer samples, 0.02 ml volume which were exposed to sunlight for four hours and not exposed, were tested.

The gas chromatogram thus obtained is shown in Fig. 15.

As shown in Fig. 15, no changes of the peaks caused

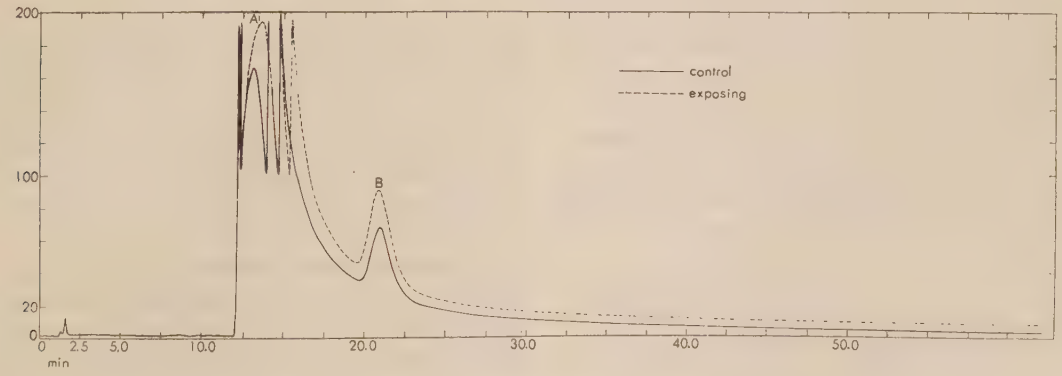


FIG. 15. Chromotogram of Beer.

by exposure to sunlight were observed, but judging from the areas of peaks A and B, beer exposing to sunlight was much more markable than the control.

The authors wish to acknowledge their indebtedness to the Shimadzu Seisakusho LTD., for measurements of the gas chromatography and to Nippon Beer Company and Kirin Beer Company for kindly supplying the samples.

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Phenolic Components in Cigarette Smoke*

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The isolation and identification of phenolic components contained in cigarette smoke, produced in constant-volume continuous smoking by means of an artificial smoking device, have been studied. Phenol, *m*-cresol and guaiacol were isolated as 3,5-dinitrobenzoates and identified by elementary analysis, mixed examination and infrared spectroscopy. Salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol and *p*-cresol were identified by paper chromatography of the phenylazobenzenesulfonic acid-dye derivatives. From the crude phenolic fraction, lauric acid was paper-chromatographically identified and a paraffin (m.p. 64–66°C) was isolated.

In previous papers[1,2,3], it has been reported that the chemical components of cigarette smoke, produced in constant-volume continuous smoking (smoking-volume, 20 ml/sec./1 cigt.; length of butts, 23 mm) of regular-size cigarettes (weight of filled cut-leaves, 1.078 g±5%; moisture content, 10.0%) by an artificial smoking device, were systematically fractionated and the isolation and identification of low-boiling nitrogenous compounds, neutral compounds, volatile acids and esters were carried out. The present communication deals with the isolation and identification of individual phenolic components contained in the phenolic fraction.

By application of a liquid chromatographic procedure with alumina columns, the purified phenolic fraction was separated into five parts by successive development and elution with petroleum ether, ether, ethyl acetate, ethyl alcohol and sodium hydroxide aqueous solution. After 3, 5-dinitro-benzoates of phenolic compounds were prepared from each eluate, the derivatives were chromatographed on silicic acid-celite columns with *n*-hexane containing 1% ether. The isolated individual derivatives were subjected to elementary analysis, mixed examination and infrared spectroscopy, and, phenol, *m*-cresol and guaiacol were identified.

The phenolic compounds which were not isolated as 3, 5-dinitrobenzoates owing to their very small quantities, were allowed to couple with *p*-diazobenzenesulfonic acid and the resulting mixture of phenyl-azobenzenesulfonic acid-dyes

* Part VIII. of a series of papers entitled "Studies on Tobacco Smoke".

1) M. Izawa and Y. Kobashi, This Bulletin, **21**, 357 (1957).

2) M. Izawa, Y. Kobashi and S. Sakaguchi, *ibid.*, **21**, 364 (1957).

3) M. Izawa and Y. Kobashi, *ibid.*, **22**, 47 (1958).

was subjected to paper chromatography. Then, salicylaldehyde, 2,5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were qualitatively identified by their R_F-values and spot colors.

From admixed components in the crude phenolic fraction, lauric acid was paper-chromatographically identified and a paraffin (m.p. 64–66°C) was isolated.

EXPERIMNTAL

I. Purification of Crude Phenolic Fraction

The crude phenolic fraction (88.21 g), separated from smoke-components of 22,000 cigarettes described in a previous paper[1], was divided into halves. One half (42.0 g) was dissolved in ether, treated with 10% sodium hydrogen sulfite aq. to remove admixed carbonyl compounds, and then extracted with 5% sodium hydrogen carbonate aq. to remove included fatty acids. The residual ethereal solution was extracted with 2N sodium hydroxide aq.. The sodium hydroxide extract was then acidified with 30% sulfuric acid aq., saturated with sodium chloride, and the solution was extracted with ether. A purified phenolic fraction (25.5 g) was obtained after evaporation of the solvent.

II. Isolation and Identification of Phenolic Compounds

Liquid-Chromatographic Separation of Phenolic Compounds: Phenylbenzensulfonic acid-dye derivatives were prepared from the purified phenolic fraction, and paper-chromatographed according to a method essentially identical with that described by Wen-Hua Chang et al.[4] (Details of this procedure wlil be mentioned in a following paragraph). Eight spots, of which four spots were estimated as guaiacol, phenol, *m*- or *o*-cresol, and *p*-cresol, were detected on the paper chromatogram. Then, the isolation of individual phenolic compounds was conducted by liquid-chromatography according to the procedure described by T. Yokotsuka[5]. After 10 ml of petroleum ether (b.p., 40–70°C) was added to 500 mg of the sample in 20 ml of ether, the sample solution was placed on the top of an alumina column ($4\phi \times 20$ cm, Merck's alumina), successively developed and eluted with 300 ml petroleum ether (b.p. 40–70°C), 400 ml ether, 600 ml ethyl acetate, 600 ml ethyl alcohol, and 500 ml 0.5% sodium hydroxide aq. under a nitrogen gas pressure of 50–350 mm.Hg. After two grams of the sample was developed by means of four trials with 500 mg, individual eluates obtained from four different organic solvents were combined respectively, and the solvents were distilled off in vacuo under passage of nitrogen gas. The ethyl acetate-eluate gave ca. 400 mg of residual liquor, and the ethyl alcohol-eluate ca. 500 mg. However, nothing was obtained from the petroleum ether- and ether-eluates. The sodium hydroxide-eluate was acidified with 10% sulfuric acid aq., extracted with ether, and dried with anhydrous sodium sulfate. After distilling-off the ether in vacuo under the passage of nitrogen gas, it yielded ca. 500 mg of residual liquor.

Diazo-, ferric chloride-, and fuchsin sulfite-reactions were positive in the ethyl acetate-, ethyl alcohol-, and sodium hydroxide-eluates. On the paper chromatograms of phenylazobenzenesulfonic acid-dye derivatives of each eluate, seven spot (R_F: 0.25, 0.38, 0.41, 0.55, 0.64, 0.70 and 0.80) were detected in the ethyl acetate-eluate, three spots (R_F: 0.12, 0.28 and 0.41) in the ethyl alcohol-eluate, and one spot (R_F: 0.28) in the sodium hydroxide one.

Isolation and Identification of *m*-Cresol and Phenol: The concentrated ethyl acetate-eluate (400 mg) was dissolved in 10 ml of anhydrous pyridine, added with 1.2 g of 3,5-dinitrobenzoyl chloride, and heated at 100°C on a water bath for 1.5 hrs. After cooling, the resulting mixture was poured into 200 ml of chilled 5% sulfuric acid aq., and the deposited crystallines were filtered, washed with water, shaked with 100 ml of 2% sodium hydroxide aq., filtered and washed with water. These treatments yielded 400 mg of a pale yellow massy crystal, which was divided into halves. Each half (200 mg) in a small quantity of ether was kneaded with 3 g of a mixture of silicic acid and celite (by wt., 1:1). After distilling-off the ether, the slurry was placed on the top of a column ($2\phi \times 20$ cm) packed with a mixture of silicic acid and celite (by wt., 1:1) containing a very small quantity of rhodamine 6G, and developed with *n*-hexane containing 1% ether under a nitrogen gas pressure of 250 mm.Hg. The first and latter half of a broad dark brown band, visible on a yellow fluorescent background under ultra violet irradiation, were eluted separately. The chromatography of 400 mg of the massy crystal gave 160 mg of a pale yellow needle crystal from the eluate of the first-half of the band and 145 mg of the crystal was obtained from the latter one.

From the first crystal, 40 mg of a pale yellow needle crystal (m.p. 159–162°C) was obtained after successive recrystallizations with 99.5% ethyl alcohol. The values

4) Wen-Hua Chang, R. L. Hossfeld and Wm. M. Sandstrom, *J. Am. Chem. Soc.*, **74**, 5766 (1952).
5) T. Yokotsuka, *J. Agr. Chem. Soc. Japan*, **27**, 276 (1953).

of elementary analyses of this compound were identical with those of the 3,5-dinitrobenzoate of an authentic *m*-cresol. Found: C, 55.23; H, 3.07; N, 9.40. Calcd. for $C_{14}H_{10}N_2O_6$: C, 55.63; H, 3.34; N, 9.27.

Mixed examination with the 3,5-dinitrobenzoates of an authentic *o*-, *m*-, and *p*-cresol (m.p.'s; 138°C, 165°C, and 189°C, respectively) showed that this derivative was that of *m*-resol. Its infrared spectrum was identical with that of an authentic *m*-cresol. The paper chromatography of phenylazobenzenesulfonic acid-dye derivatives, prepared after the esterification of this derivative, gave only one spot (R_F: 0.41) equivalent to *m*-cresol. The presence of *m*-cresol was confirmed by the above-mentioned facts.

On the other hand, recrystallizations of the second crystal gave 40 mg of a white needle crystal (m.p. 143–145°C), of which values of elementary analyses were identical with those of 3,5-dinitrobenzoate of an authentic phenol. Found: C, 54.26; H, 2.85; N, 9.31. Calcd. for $C_{13}H_8N_2O_6$: C, 54.17; H, 2.80; N, 9.72.

The m.p. of this crystal was not depressed upon admixture with an authentic specimen and the two crystals had the same infrared spectrum. Consequently, phenol was identified.

Isolation and Identification of Guaiacol: Pale yellow massy crystals of 3,5-dinitrobenzoate (150 mg) were prepared from the concentrated ethyl alcohol-eluate (300 mg). The derivatives were separated into three bands by the same liquid-chromatography as described above. These bands were eluted and distilled off the solvent. A yellow viscous substance obtained from the first band, was repeatedly recrystallized from 99.5% ethyl alcohol, and identified as the 3,5-dinitrobenzonate of *m*-cresol by infrared spectroscopy.

From the second band, a white needle crystal (35 mg) of m.p. 143–145°C was obtained. The m.p. of this crystal was not depressed upon admixture with the 3,5-dinitrobenzoate of an authentic phenol (m.p. 145°C), and infrared spectra of the two specimens were found to be identical. Therefore, this compound was proved as phenol.

The eluate from the third band gave a pale yellow crystal (40 mg), which was recrystallized from 99.5% ethyl alcohol until 10 mg of a pale yellow needle crystal (m.p. 140–141°C) was obtained. The values of elementary analyses of this crystal were identical with those of the 3,5-dinitrobenzoate of an authentic guaiacol (m.p. 141°C), and the m.p. of the former crystal was not depressed upon admixture with the latter crystal; the infrared spectra of the two specimens were also identical. From these evidences, guaiacol was identified. Found: C, 53.10; H, 3.34; N, 8.49. Calcd. for $C_{14}H_{10}N_2O_7$: C, 52.84; H, 3.17; N, 8.80.

III. Paper-chromatographic Identification of Salicylaldehyde, 2,5-Dimethylphenol, *m*-Ethylphenol, and *p*-Cresol

As it was estimated that several phenolic compounds other than *m*-cresol, phenol and guaiacol may be present

TABLE I. PAPER CHROMATOGRAPHY OF PHENYLAZOBENZENESULFONIC ACID-DYE DERIVATIVES OF PHENOLIC COMPOUNDS IN CIGARETTE SMOKE

Compound	Authentic Specimen		Specimen from Smoke	
	R_F	Color	R_F	Color
Guaiacol*	0.12	Orange	0.12	Orange
Phenol*	0.25	Yellow	0.25	Yellow
Salicylaldehyde	0.38	Yellow	0.38	Yellow
m-Cresol*	0.41	Yellow	0.41	Yellow
o-Cresol	0.41	Yellow	—	—
2, 5-Dimethylphenol	0.55	Orange Yellow	0.55	Orange Yellow
?	—	—	0.64	Orange Yellow
m-Ethylphenol	0.71	Yellow	0.70	Yellow
3, 5-Dimethylphenol	0.72	Yellow	—	—
o-Phenylphenol	0.74	Orange Yellow	—	—
p-Cresol	0.81	Pink	0.80	Pink

Remarks:
Paper...Whatman No. 1 paper impregnated with 4% $NaCO_3$ aq.
Solvent...*sec*-butanol: 2% $NaCO_3$ aq. (by vol. 1:1)
Development...Descending technique, room temp., 15 hrs.
*...Identified as 3,5-dinitrobenzoates.

in very small amounts, paper-chromatographic identification according to a procedure similar to that of Wen-Hua Chang et al.[4] was carried out employing their phenylazobenzenesulfonic acid-dye derivatives.

The purified phenolic fraction (ca. 50 mg) as described in the first paragraph, was dissolved in 2N-sodium hydroxide aq. (0.5 ml), added *p*-diazobenzenesulfonic acid (ca. 10 mg), acidified with 10% hydrochloric acid aq., and diluted with ethyl alcohol. A very small quantity of the thus prepared solution was descendingly developed with a solvent of *sec*-butanol: 2% sodium carbonate aq. (by vol. 1:1) on a Whatman No. 1 paper at room temperature for about 15 hrs. Before use, the paper was air-dried after spraying with 4% sodium carbonate aq..

The results are shown in Table I. Salicyladehyde, 2,5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were paper-chromatographically identified by the judgment of R_F-values and color of spots. However, a compound, showing an orange yellow color and R_F 0.64 on the paper chromatogram, was not identified.

IV. Paper-Chromatographic Identification of Lauric Acid

In the former paragraph it has already been described that admixed fatty acids in the crude phenolic fraction were extracted with 5% sodium hydrogen carbonate aq.. This extract was acidified with 10% sulfonic acid aq. and extracted with ether. The concentrated ether extract was paper-chromatographed by a reverse phase-technique for higher fatty acids according to the manner of Y. Inoue et al.[6].

A Whatman No. 1 paper (2×30 cm) was impregnated with petroleum hydrocarbon (b.p. 140–165°C) and the excess solvent was removed by slight pressing with two sheets of filter paper. A minute portion of petroleum hydrocarbon solution of the sample was quickly spotted on the paper, and ascendingly developed with a solvent of methanol: petroleum hydrocarbon (by vol. 4:1) at 30°C in a closed chamber. After complete development, only one spot (R_F, 0.74) equivalent to that of lauric acid, was detected on the paper sprayed with an alkaline alcoholic solution of 0.2% bromocresol green. From this experiment it was confirmed that lauric acid is present as a higher fatty acid admixed in the crude phenolic fraction.

V. Isolation of Paraffin

The ether-layer, remaining after the extraction of the crude phenolic fraction with 2N-sodium hydroxide aq. (already described in the paragraph "Purification of Crude Phenolic Fraction"), gave an orange yellow viscous substance after solvent-evaporation. This substance was repeatedly treated with 99.5% ethyl alcohol, and a paraffin (m.p. 64–66°C), insoluble in 99.5% ethyl alcohol, was obtained. This paraffin was estimated to be identical with the paraffin reported in a previous paper[2].

6) Y. Inoue and M. Noda, *J. Agr. Chem Soc. Japan*, **26**, 634 (1952).

DISCUSSION

Recent papers concerning phenolic components in cigarette smoke are presented by C. H. Rayburn et al.[7] and B. T. Commins et al.[8,9].

C. H. Rayburn et al. determined the quantities of phenol, guaiacol, *m*-, and *o*-cresol in the smoke of cigarettes prepared from each of the four types of tobacco leaves. The smoking conditions were two-sec. puffing twice per minute, 35 ml/two sec. volume, and 23-mm butt. In their experiments, coupled derivatives between phenols and diazotized *p*-nitroaniline were paper-chromatographed and each derivative was eluted on a separated strip of paper and spectrophotometrically determined.

On the other hand, in studies on the smoke of Virginia cigarettes, B. T. Commins et al. spectrophotometrically determined phenol, *o*-, *m*-, *p*-cresol, α-naphthol, β-naphthol, catechol, resorcinol, and hydroquinone by converting them into methylesters with dimethyl sulfate.

In the present work, the isolation and identification of phenolic compounds as crystalline derivatives were attempted and the presence of phenol, *m*-cresol and guaiacol was confirmed in a crystalline state of their 3, 5-dinitrobenzoates. Phenols, not isolated as crystalline derivatives owing to their very small quantity, were studied by paper chromatography of their phenylazobenzenesulfonic acid-dye derivatives; and, salicylaldehyde, 2, 5-dimethylphenol, *m*-ethylphenol, and *p*-cresol were qualitatively identified. Althogh the presence of another unidentified substance was observed, spots equivalent to α-

7) C. H. Rayburn, W. R. Harlan and H. R. Hanmer, *Anal. Chem.*, **25**, 1419 (1953).

8) B. T. Commins and A. J. Lindsey, *Analytica Chimica Acta*, **15**, 557 (1956).

9) B. T. Commins and A-J. Lindsey., *Brit. J. Cancer*, **10**, 504 (1956)

naphthol, β-naphthol, catechol, resorcinol, and hydroquinone, were not detected in the authors' sample. The presence of *o*-cresol, already reported by other authors, was not confirmed because the amount obtained was very small and the R_F-value was same as that of *m*-cresol.

The phenols, whose presence could not be confirmed in the authors' sample, will be investigated in further studies concerning the smoke of Japanese cigarettes.

Acknowledgment The authors wish to express their sincere thanks to Prof. Y. Obata of Hokkaido University for his guidance and encouragement in the course of this work. They are also grateful to Director T. Nakashima and Chief I. Ōnishi of this Research Institute for their encouragement and support.

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Free Amino Acids in Cigarette Smoke (I)*

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Free amino acids in the cigarette smoke, produced in constant-volume continuous smoking by the use of an artificial smoking-device, have been paper-chromatographically studied, and twelve amino acids, i.e., α-alanine, β-alanine, glycine, glutamic acid, glutamine, serine, γ-amino butyric acid, valine, leucine, aspartic acid, proline, and ornithine(?) were qualitatively identified. Besides these amino acids, the presence of the other two unidentified ninhydrin-positive substances was observed. The presence of ten amino acids, other than glutamic acid and glutamine, has not yet been reported in the literature concerning tobacco smoke.

Up to the present, a number of papers dealing with amino acids in tobacco leaves have been reported by many authors[1,2,3,4]. Concerning tobacco smoke, however, only one paper is available. This is the paper recently published by D. A. Buyske et al.[5], who paper-chromatographically identified glutamic acid and glutamine, together with the other four unidentified ninhydrin-positive substances, in cigarette smoke.

The present paper deals with the qualitative identification of 12 separate amino acids contained in the smoke of Japanese blended cigarettes by means of paper chromatography.

Regular-size cigarettes were continuously smoked under a condition of constant-volume smoking by an artificial smoking device. The somke was collected in a train of absorbing bottles and the resulted smoke solution was purified by shaking with ether and by ion-exchange column chromatography. The purified

* Part IX. of a series of papers entitled "Studies on Tobacco Smoke"

1) H. Michl und H. Kuhn, *Fachl. Mitteil. Österr. Tabakregie*, **Jg. 1954, Hft. 1**, 10 (1954).

2) F. Habeler, H. Kuhn und H. Michl, *ibid.*, **Jg. 1955, Hft. 1**, 12 (1955).

3) S. Ranjan and M. M. Laloraya, *Nature*, **177**, 235 (1956).

4) M. Nagasawa and K. Yamamoto, *Sci. Papers Centr. Res. Inst. Japan Monop. Corp.*, **No. 96**, 50 (1956).

5) D. A. Buyske, J. E. Flowers, Jr., P. Wilder, Jr. and M. E. Hobbs, *Science*, **124**, 1080 (1956).

sample solution was subjected to two-dimensional paper chromatography, and twelve amino acids, i.e., α-alanine, β-alanine, glycine, glutamic acid, glutamine, serine, γ-amino butyric acid, valine, leucine, aspartic acid, proline and ornithine (?) were qualitatively identified. Furthermore, the presence of the other two ninhydrin-positive substances was observed on the paper chromatograms.

EXPERIMENTAL

Sample Cigarettes Five hundred regular-size cigarettes, blended with U.S. Flue-cured 10.2%, Japanese Flue-cured 66.7%, Japanese Native "Matsukawa-ha" 18.0%, and Indian Native tobacco 5.1%, were employed in this experiment. Their moisture content, nicotine content and the average weight per cigarette were 10.51% (in wet basis), 2.39% (in dry basis) and 1.041 g, respectively.

Smoke Collection and Preparation of Sample Solution: An artificial smoking device and a smoke collection train were similar to those reported in a previous paper[6]. Both of these were composed of a set of five cigarette holders, a flask containing water (1.5 lit.) and ether (1.2 lit.), and six washing bottles containing water. Three empty bottles were inserted amongst each unit. The flask was cooled with a freezing mixture of ice and sodium chloride. Every cigarette was continuously smoked, leaving 25 mm-butts, at the constant smoking volume of 20 ml/sec./1 cigarette. To maintain this constant smoking volume, a flow meter equipped with two by-pass cocks, was inserted between the set of cigarette holders and the first empty bottle. The suction was regulated by the readings of the meter caused by occasional opening of the by-pass cocks. After the smoking of 500 cigarettes, about two lit. of aqueous smoke-solution was obtained.

To remove considerable quantities of admixed alkaloids, amines, acids, paraffin-like compounds, colored substances and the other impurities, the aqueous smoke-solution was repeatedly washed with ether. Then, the solution was adjusted to pH 11 with ammonia, extracted five times with ether by shaking, and filtered. The filtrate was concentrated in vacuo to almost dryness at 50°C, and the residue was dissolved in a small amount of water. After repetition of concentration and filtration, a purified sample solution was obtained.

Treatment of Smoke Solution by Ion-Exchange Resins: The purified sample solution was allowed to pass through a column (2.6ϕ × 25 cm) packed with Dowex 50-X8 (200–400 mesh) at the flow-rate of 10–20 drops per min.. The liquor, freely passed through the resin, was re-chromatographed on another similar column. The adsorbed resin on the column was washed with water and eluted with 500 ml of 3N-hydrochloric acid. The eluates were concentrated to almost dryness, the residue was dissolved in water, and filtered. These concentration and filtration procedures were repeated several times.

An aliquot of the filtrate was paper-chromatographed with a solvent of butanol : acetic acid : water (4 : 1 : 5), and more than nine ninhydrin-positive substances were detected. However, the presence of consistent impurities, inhibiting the complete separation of individual amino acids, was observed on the paper chromatograms; hence the entire filtrate was chromatographed through a column (2.0ϕ × 30 cm) packed with IRA 400 (20–50 mesh). After unadsorbed substances on the column were removed by washing with water, 500 ml of 0.1N-hydrochloric acid was allowed to pass through the resin. The eluates were evaporated to almost dryness and diluted to 10 ml with water. Thus, a purified sample solution, rich in amino acids, was obtained.

This solution practically contained no impurities. That is, each paper chromatogram was respectively sprayed with ninhydrin, Dragendorf's, Nessler's, iodine, or p-amino-benzoic acid-cyanogen bromide reagent and indicated the absence of compounds other than amino acids.

Furthermore, for detailed identification of the amino acids appearing close to the starting point on the paper chromatograms, a portion of the above-mentioned sample solution was further treated on a column of Dowex 2-X8 (50–100 mesh).

Paper-chromatographic Identification of Free Amino Acids:

The identification of free amino acids was carried out by the two-dimensional descending paper chromatography according to the procedure of A.L. Levy and D. Chung[7].

Chromatographic cabinet: A cabinet, made of stainless steel, with a glass window at one end (70 × 70 × 25 cm) was employed.

Filter paper: Whatman No. 1 filter paper.

Development: After the first dimensional development was carried out with a solvent of butanol : acetic acid : water (4 : 1 : 5), the paper was sprayed with a buffer solution of boric acid-sodium hydroxide (pH, 8.3) and dried. The second dimensional development was carried out with a solvent of m-cresol : phenol : boric acid-

6) M. Izawa and Y. Kobashi, This Bulletin, **21**, 357 (1957).

7) A. L. Levy and D. Chung, *Anal. Chem.*, **25**, 396 (1953).

sodium hydroxide buffer of pH 8.3 (ratio, 60g: 30g: 15ml). After the development, a 0.2% ninhydrin-butanol solution was sprayed and the paper was dried at 80–90°C.

Identification: The paper chromatograms of the smoke-sample were compared with those of 21 authentic amino acids developed under the same conditions. To ascertain the identification, a very small quantity of each authentic specimen of amino acid which was estimated to be present in the smoke sample, was separately added to the smoke sample, by twos and threes, and many chromatograms were run on separate sheets. The spots appearing on the paper chromatograms were compared with those of the untreated smoke sample.

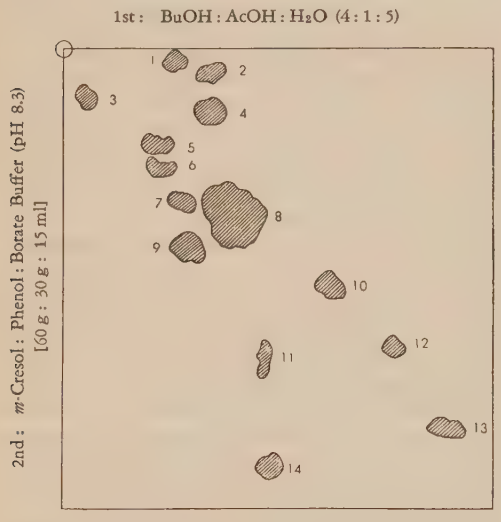

FIG. 1. Two-Dimensional Paper Chromatography of Amino Acids in Cigarette Smoke.

1.	Aspartic acid	+	8.	α-Alanine	+ + + + +
2.	Glutamic acid	+ +	9.	β-Alanine	+ + +
3.	Ornithine (?)	+	10.	Unidentified	+
4.	Glutamine	+ +	11.	γ-Amino butyric acid	+ +
5.	Serine	+ +	12.	Valine	+ +
6.	Glycine	+ + +	13.	Leucine	+ +
7.	Unidentified	+ +	14.	Proline	+

Remark: +...Size and color-intensity of spots

The following evidences were obtained from the color intensity and size of spots on the paper chromatograms. As shown in Fig. 1, twelve amino acids were qualitatively identified. The most abundant was α-alanine; glycine and β-alanine were in the second place; glutamic acid, glutamine, serine, γ-amino butyric acid, valine and leucine existed in small quantities; aspartic acid, proline and ornithine(?) were detected in very small amounts; and, the presence of the other two unidentified ninhydrin-positive substance was observed.

DISCUSSION

H. Michl et al.[1] and M. Nagasawa et al.[4] reported that proline, α-alanine and γ-amino butyric acid in leaf tobacco exist in most abundant amounts. The present studies on the cigarette smoke indicated that α-alanine showed the same trend, but the amount of γ-amino butyric acid was small and proline was extremely small. Concerning glutamic acid, glutamine, asparagine and aspartic acid existing in comparatively large amounts in the leaves, the first two were small, and aspartic acid was very small, but asparagine was not detected in this study. Phenylalanine and histidine detected in the leaves, could not be found in this experiment. On the other hand, leucine and ornithine, of which presence in the leaves is uncertain, were detected in the present authors' smoke sample.

D. A. Buyske et al.[5] have found six ninhydrin-positive substances in cigaratte smoke, of which two were identified as glutamic acid and glutamine. Their analysis seems to suggest that the contents of these two amino acids in cigarette smoke may be comparatively larger than those of other amino acids. In the present authors' smoke sample, however, the contents of both amino acids were smaller than those of α-alanine, β-alanine or glycine.

Acknowledgment The authors wish to express their sincere thanks to Prof. Dr. Y. Obata of Hokkaido University for his guidance and encouragement in the course of this work. They are also grateful to Director T. Nakashima and Chief I. Ōnishi of this Research Institute for their encouragement and support.

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Free Amino Acids in Cigarette Smoke (II)*

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Free amino acid contained in the smoke of seven different cigarettes, respectively made from a single variety of tobacco, have been quantitatively determined by paper chromatography and colorimetry. Fourteen amino acids (α-alanine, proline, glycine, glutamic acid, glutamine, β-alanine, serine, γ-amino butyric acid, aspartic acid, valine, leucine, ornithine, threonine, and phenylalanine) and three ninhydrin-positive substances were detected. Of these acids α-alanine was most abundant (10.5–268.2γ/1 cigt.) and proline (5.5–25.1γ), glycine (4.7–22.5γ), etc. followed in this order. In general, the smoke of sun-cured or bulk-sweated tobaccos contained more kinds and more amounts of amino acids than that of flue-cured tobaccos. In another experiment, it was found that about 15% of artificially added mono-sodium glutamate is transferred into cigarette smoke.

Recently, D. A. Buyske et al.[1] determined quantitatively 10 μg of glutamic acid and 7 μg of glutamine per one cigarette in their study concerning the constituents of smoke puffed from a 50/50 blend of bright and burley tobaccos. However, any other studies concerning amino acids in tobacco smoke have so far not been available.

In the present authors' previous paper[2], it has been reported that 12 amino acids were qualitatively identified in the smoke of blended cigarettes by paper chromatography. The present paper deals with the detection and quantitative determination of amino acids in the smoke of seven different types of cigarettes respectively made from a single variety of leaf tobacco; and, an experiment on the amino acid-transfer into smoke from the cigarettes spraped with sodium glutamate solution.

EXPERIMENTAL

I. Quantitative Determination of Free Amino Acids in Cigarette Smoke

Sample Cigarettes and Smoke Collection: The sample cigarettes were respectively made from a single variety of leaf tobacco as shown in Table 1. The cut-width of filled cut-leaves was 0.5 mm and the length of the cigarettes was 70 mm. Each cigarette was filled with cut-leaves in a hardness suitable for comfortable smoking. Consequently, the cigarette-weight of each variety was different, respectively, as shown in Table I.

Table I. Varieties of Leaf Tobaccos and Weight of Filled Cut-Leaves in One Cigarette

Variety of Leaf Tobacco	Filled Cut-Leaves per One Cigarette (g)
U. S. Flue-cured	1.1163
Japanese Flue-cured	1.0033
Japanese Native, "Matsukawa-ha"	0.7643
Japanese Native, "Daruma-ha"	0.7098
Japanese Native, "Nambu-ha"	0.9332
Japanese Burley	0.7498
Turkish, Izmir-B	1.2033

Two hundred cigarettes of each variety were automatically smoked, leaving 25 mm-butts, in the same manner as described in a previous paper[2].

Preparation of Sample Solution: Automatic smoking of the seven varieties of cigarettes gave aqueous smoke solutions of 1.5–2 lit., respectively. Each smoke solution was shaked with ether three times. After the residual aqueous solution was added barium hydroxide

* Part X. of a series of papers entitled "Studies on Tobacco Smoke"

1) D. A. Buyske, J. E. Flowers, Jr., P. Wilder, Jr. and M. E. Hobbs, *Science*, **124**, 1080 (1956).

2) M. Izawa, Y. Kobashi and M. Taki, This Bulletin, **23**, 198–200 (1959).

(15 g) and shaked with ether five times, the aqueous layer was neutralized to pH 5–6 with 1N-sulfuric acid. The precipitated barium sulfate was filtered off and the filtrate was concentrated to almost dryness in vacuo. The concentrated solution was dissolved in a small amount of water; and, developed and eluted on a Dowex 50–X4 (100 ml, 20–50 mesh) column with 2N-ammonia (300 ml). After the eluate was evaporated to almost dryness in vacuo to remove the ammonia, the residue was dissolved in a small amount of water and filtered. The filtrate was chromatographed with Dowex 2–X8 (60 ml, 50–100 mesh) and 0.2N-hydrochloric acid (500 ml). After the eluate was concentrated in vacuo to remove the hydrochloric acid, it was dissolved in a small amount of water and filtered. The filtrate was subjected to one more respective treatment with Dowex 50–X4 (60 ml) and Dowex 2–X8 (45 ml). Thus, seven concentrated sample solutions of 10 ml were obtained.

On the other hand, to investigate the amount of each amino acid recoverable in ion-exchange resin chromatography, seven amino acids were chosen, and 50 mg of each authentic amino acid in 10 ml of water was subjected to the same ion-exchange resin treatments as described above. The yields were calculated from the amino-nitrogen contents of the solutions both before and after treatments. The results are given in Table II.

Table II. Average Recovery of Amino Acids by Ion-Exchange Resin Chromatography

Amino Acid	Amino-N fed	Amino-N recovered			
		Dowex 50–X4 column		Dowex 2-X8 column	
	(mg)	(mg)	(%)	(mg)	(%)
Aspartic acid	5.26	4.60	87.38	5.00	95.06
Glutamic acid	4.76	4.83	101.47	4.44	93.33
Glycine	9.33	9.03	96.78	7.68	82.32
Serine	6.67	6.36	95.35	7.36	110.34
α-Alanine	7.86	7.50	95.42	7.13	90.71
γ-Amino butyric acid	6.79	6.78	99.82	6.43	94.71
Proline	6.09	6.14	100.87	6.01	98.83

Paper-chromatographic Identification of Free Amino Acids:

For identification of free amino acids in the above-mentioned seven smoke-solutions, a two-dimensional descending paper chromatography was carried out in a manner essentially the same as that described by M. Wolfe[3].

Chromatographic cabinet: A cabinet, made of stainless steel, with a glass window at one end (70×70×25 cm) was employed.

Filter paper: Toyo No. 51 filter paper (50×55 cm)

Development: After the first dimensional development was carried out with a solvent of *n*-butanol: methyl ethyl ketone: water: 17N-ammonia (5:3:1:1 by vol.) at 21°C for 20–22 hrs., the paper was dried at 40°C for three hrs.. The second dimensional development was then run with a solvent of *n*-butanol: acetic acid: water (4:1:5 by vol.) for 8–12 hrs. After development the paper was dried and treated with a spray reagent.

Spray reagents: (1) 0.2% ninhydrin-butanol solution. Heating at 90°C for coloration; (2) Solution prepared by heating and filtering of a mixture of isatin, zinc acetate, pyridine, and *iso*-propyl alcohol (1 g: 1.5 g: 1 ml: 100 ml). Heating at 90°C for coloration[4]. The latter reagent gave good results especially for the detection of proline (color—blue) and alanine (color—grayish violet).

Identification: The paper-chromatograms of the smoke-samples were compared with those of authentic amino acids developed under the same conditions. To ascertain this identification, a very small quantity of each authentic specimen of amino acids which were estimated to be present in the smoke samples, was separately added to the smoke samples, by ones and twos, and many chromatograms were run on separate sheets. The spots appearing on the paper chromatograms were compared with those of the untreated smoke samples.

The paper chromatograms of seven smoke samples and a mixture of authentic amino acids are shown in Fig. 1.

Quantitative Determination of Amino Acids by Paper Chromatography and Colorimetry:

After the paper-chromatographic development was carried out according to the method of M. Wolfe[3], H.

3) M. Wolfe, *Biochim. et Biophys. Acta*, **23**, 186 (1957).

4) J. Barrollier, J. Heilman und E. Watzke, *Zeitschr. f. physiol. Chem.*, **304**, 21 (1956).

1. Aspartic acid
2. Glutamic acid
3. Glutamine
4. Ornithine
5. Glycine
6. Serine
7. β-Alanine
8. α-Alanine
9. γ-Amino butyric acid
10. Proline
11. Valine
12. Threonine
13. Phenylalanine
14. Leucine

U. Unidentified ninhydrin-positive substance

FIG. 1. Paper Chromatograms of Free Amino Acids in the Smoke of Cigarettes Made from Respective Single Variety of Leaf Tobacco

Remarks... ↓, first dimension; *n*-butanol : methyl ethyl ketone : 17 N-ammonia (5 : 3 : 1 : 1 by vol
←, second dimension; *n*-butanol : acetic acid : water (4 : 1 : 5 by vol)

Rosen's modified ninhydrin-colorimetric procedure[5] in which the color intensity of spots on the paper chromatogram is measured, was applied for the determination of amino acids. The method of L. Fowden[6] was also referred in these determinations.

A portion of the smoke sample (0.05 ml) was micropipetted and two-dimensionally paper-chromatographed. After drying at 40°C for three hrs., the paper was slightly sprayed with 0.1% ninhydrin-acetone solution and heated at 80–90°C. Colored spots on the paper chromatogram were cut off. Each paper strip was slipped into a test tube; and then 0.0002M-sodium cyanide--acetate buffer (0.5 ml, a buffer of pH 5.3–5.4; prepared with 360 g sodium acetate crystal, 67 ml acetic acid and 933 ml water) and 3% ninhydrin-cellosolve solution (0.5 ml) were added. The mixture was heated on a water bath at 100°C for 15 min.; a solution of acetone : water (1 : 1 by vol.) was added and shaked vigorously. After cooling to room temperature, the solution was filled up to 10 ml. with a mixture of acetone and water. By a Beckman B-type spectrophoto-

Table III. Average Recovery of Amino Acids on Paper Chromatography

Amino acid	Average Recovery (%)
Aspartic acid	102.0
Glutamic acid	106.5
Glycine	86.0
Serine	82.7
α-Alanine	98.7
γ-Amino butyric acid	100.2
Proline	90.3

Table IV. Contents of Free Amino Acids in the Smoke of Cigarettes Made from Respective Single Variety of Leaf Tobacco

	Amino Acid	Flue-cured		Japanese Native			Burley	Turkish
		U. S.	Japanese	"Matsukawa-ha"	"Daruma-ha"	"Nambu-ha"	Japanese	Izmir-B
1.	Aspartic acid*	12.2	+	+	3.0	10.6	8.1	12.7
2.	Glutamic acid*	22.0	24.8	2.7	3.5	23.6	27.0	23.5
3.	Glutamine							
4.	Ornithine	–	–	–	–	3.7	+	9.1
5.	Glycine*	16.8	4.7	7.5	13.0	14.5	14.0	22.5
6.	Serine*	2.0	+	+	2.5	2.2	5.6	11.2
7.	β-Alanine	6.0	2.4	+	3.5	4.5	5.5	5.3
8.	α-Alanine*	54.5	23.5	10.5	59.5	122.9	268.2	242.3
9.	γ-Amino butyric acid*	12.0	7.1	+	+	3.1	15.5	15.7
10.	Proline*	13.8	11.2	5.5	19.5	7.5	7.5	25.1
11.	Valine	+	+	+	+	+	8.0	10.2
12.	Threonine	–	–	–	–	?	?	?
13.	Phenylalanine	–	–	–	–	+	+	+
14.	Leucine	+	+	?	?	+	7.1	7.0
U.	Unidentified ninhydrin-positive substance (number)	1	1	1	2	3	2	2
Amino-N in the smoke of cigts. (mg)		5.90	3.78	2.19	5.09	11.66	13.93	12.76
Amino-N in the smoke of 100 g burnt portion of cigts. (mg)		4.11	2.93	2.23	4.58	9.72	14.45	8.25
Total N in cigts. (%)		1.74	1.63	2.16	1.96	3.67	2.37	2.18

Remarks: (1) Contents of each amino acid are tabulated in γ per one cigarette.
(2) Asterisked amino acids were corrected according to the recoveries shown in Table II and III.
(3) Because glutamic acid and glutamine gave an overlapped spot on the paper chromatograms, both amino acids were determined as glutamic acid.
(4) + indicates "comparatively small" and ? indicates "comparatively very small or trace".

5) H. Rosen, *Arch. Biochem. Biophys.*, **67**, 10 (1957).
6) L. Fowden, *Biochem. J.*, **48**, 327 (1951).

Table V. Transfer of Amino Acid into Cigarette Smoke
(per 300 cigarettes)

Total wt. of employed cigts.	(g)	322.3
Total wt. of burnt portion of cigts.	(g)	207.2
Amts. of added glutamate in burnt portion of cigts. (as amino-N)	(mg)	
Cigts. sprayed with glutamate		51.5
Cigts. not sprayed with glutamate		0.0
Amts. of amino acids in smoke (as amino-N)	(mg)	
Cigts. sprayed with glutamate (I)		18.29
Cigts. not sprayed with glutamate (II)		10.55
Difference between (I) and (II)		7.74
Ratio of amino acid-transfer into smoke	(%)	15.03

Remark: Cigarettes of 70 mm were smoked leaving 25-mm butts.

meter, measurement of absorbance was made at 570 mμ (proline at 440 mμ) under the absorbance between 0.2 and 0.8.

The quantity of each amino acid was calculated from the standard absorbance-concentration curves, which were previously prepared with 0.1, 0.2, or 0.5 ml of 0.01% aqueous solution of each authentic amino acid by H. Rosen's modified ninhydrin-colorimetric procedure[5]. Furthermore, for correcting these values, the following blank determinations were carried out: Uncolored paper strips of the same size, neighboring colored spot positions, were cut off from the filter paper, and, the absorbance measurements and calculations were conducted in the same manner as described above.

On the other hand, 0.02%, 0.04% and 0.06% aqueous solutions of each authentic amino acid were prepared. From these solutions, 0.05 ml was taken respectively and an experiment on the recovery of amino acids in the above-mentioned paper chromatography itself was carried out. The results obtained are shown in Table III. From these recoveries, corrections were made on the determined amounts of amino acids in the seven smoke samples described above.

The results given in Table IV were obtained according to the corrections made on the recoveries of the ion-exchange treatments as described in Table II. The contents of amino-nitrogen in the smoke and the total nitrogen contents of each cigarette are also tabulated in Table IV.

II. An Experiment on Amino Acid-Transfer into Cigarette Smoke

Regular size U.S. flue-cured cigarettes (weight of cut leaves per one cigarette—1.07 g±5%) were employed and the following experiment was undertaken in investigate the amount of amino acid transferred into cigarette smoke.

Sample cigarettes: (1) Cigarette made from cut-leaves sprayed with 50% mono-sodium glutamate solution by 0.3% per weight of cut-leaves as mono-sodium glutamate.

Smoking and analytical procedures: Applying the same procedure as described in the preceding paragraph, 300 cigarettes of both kinds were automatically smoked and the collected smoke solutions were treated by ion-exchange resins. Amino-nitrogen in both smoke solutions was determined by Van Slyke's procedure.

Results: The average values obtained from several trials are tabulated in Table V.

DISCUSSION

(1) As shown in Fig. 1, 14 amino acids were detected in the smoke of seven different cigarettes respectively made from a single variety of tobacco. All of the amino acids, already reported to be contained in the smoke of blended cigarettes[2], were also found in the present work, and, the other two, threonine and phenylalanine, were first detected in some of the cigarettes tested in the present study.

Furthermore, the presence of 1–3 unidentified ninhydrin-positive substance was proved. One of them, situated near the proline spot on the paper chromatogram, exists in a considerable amount in all samples, and, the other one, situated near γ-amino butyric acid, showed a characteristic figure that it exists only in the smoke of sun-cured or bulk-sweated tobaccos.

(2) According to the values obtained from determinations (Table IV), throughout the seven samples, α-alanine was most abundant (10.5–268.2 γ/1 cigt.), its contents being 23.5–54.5 γ in

flue-cured, 10.5–122.9 γ in Japanese native, 242.3 γ in Turkish, and 268.2 γ in Burley tobacco. Proline (5.5–25.1 γ), glycine (4.7–22.5 γ), and glutamic acid (2.7–27.0 γ) followed. The contents of β-alanine, serine, γ-amino butyric acid and aspartic acid were comparatively small. Valine and leucine were found to exist in small amounts in case of the smoke of Burley and Turkish tobaccos and in a very small or in trace quantities in other tobaccos. Ornithine, threonine and phenylalanine existed in very small or in trace quantities only in Burley, Turkish and "Nambu-ha".

(3) As a general trend, the smoke of sun-cured or bulk-sweated tobaccos contain more kinds and more quantities of amino acids than that of flue-cured tobaccos. That is, the contents of amino-nitrogen in the smoke of a 100 g burnt portion of cigarette were as follows: Burley 14.45 mg, Turkish 8.25 mg, bulk-sweated "Nambu-ha" 9.72 mg, sun-cured "Daruma-ha" 5.58 mg, and, U. S. and Japanese flue-cured tobaccos 4.11 mg and 2.93 mg. In spite of sun-cured tobacco, "Matsukawa-ha" showed a comparative smaller content, 2.23 mg, resembling that of flue-cured tobaccos. In Japan this "Matsukawa-ha" tobacco is more widely utilized in practical cigarette-blends than other Japanese native tobaccos, because its superior properties are harmonious with flue-cured tobaccos and serve to improve the aroma and taste of Japanese cigarettes. Thus, it is very interesting that the above analytical data agree closely with this practical evidence.

(4) It is a very interesting phenomenon that involatile substances transfer into the cigarette smoke. Recently, E. C. Cogbill et al.[7] presented an experimental evidence that even inorganic metallic salts are found in tobacco smoke. In the present experiment concerning involatile mono-sodium glutamate, it was shown that about 15% of artificially added glutamate transfer into smoke and the addition of glutamate to cigarettes gives a mellow taste to smoke. It is considered that this phenomenon is valid for improvement of the smoking taste of cigarettes.

Acknowledgment The authors wish to express their sincere thanks to Prof. Dr. Y. Obata of Hokkaido University for his guidance and encouragement throughout the course of this work. They are also grateful to Director T. Nakashima and Chief I. Ōnishi of this Research Institute for their encouragement and support.

7) E. C. Cogbill and M. E. Hobbs, *Tobacco*, **144** (No. 19), 24 (1957)

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 207~213, 1959]

Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

I. Basic Properties and Effects of Several Inhibitors*

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In order to study the properties of crystalline phosphoglyceric acid mutase, the polarimetric method was employed for the direct measurement of the enzyme activity. As a result, it was found that the enzyme was inhibited by various metallic ions, chelating agents and phosphoryl enolpyruvate, but not influenced by SH-inhibitors. In addition, fluoride was found to inhibit the enzyme activity in a special manner. Some observations on the basic properties are also presented.

INTRODUCTION

Phosphoglyceric acid mutase discovered by Meyerhof and Kiessling[1] catalyzes the following reaction, D-3-phosphoglyceric acid $\rightleftharpoons$ D-2-phosphoglyceric acid, and plays an important role in glycolysis. Sutherland et al.[2] showed that 2,3-diphosphoglyceric acid acted as the coenzyme. Various properties of this enzyme, however, were not investigated in detail until comparatively recent years, since the highly purified preparation of PGA mutase** had not been obtained. Recently Rodwell et al. have first reported the crystallization of PGA mutase from baker's yeast[3], and described the enzymological and molecular properties of the crystalline enzyme from yeast and also those of the highly purified enzyme from rabbit skeletal muscle[4,5]. Hereupon, knowledge of PGA mutase has first been introduced. Pizer et al.[6] have also reported studies on the mechanism of the reaction which is catalyzed by muscle PGA mutase. But these studies utilized enolase in the investigation of the mechanism of the PGA mutase reaction, and therefore influences caused by the properties of enolase can not be disregarded. Especially, in inhibition experiments, effects of inhibitors on enolase must be considered. Then it is necessary that the mutase activity should be directly measured without the addition of enolase. For this purpose, the polarimetric measurement is suitable for the enzyme assay. The purpose of this present communication is to describe some of the more pertinent properties of the crystalline enzyme obtained from baker's yeast according to a modification of the procedure of Rodwell et al.[3]. In our studies the enzyme activity was directly determined by the polarimetric method.

MATERIALS AND METHODS

DL-2PGA was synthesized by oxidation of sodium β-

* Presented at the Meeting of the Agricultural Chemical Society of Japan, Kyoto, May 3, 1958.

1) O. Meyerhof and W. Kiessling, *Biochem. Z.*, **276**, 239 (1935); *ibid.*, **280**, 99 (1935).

2) E. W. Sutherland, T. Posternak and C. F. Cori, *J. Biol. Chem.*, **179**, 501 (1949); *ibid.*, **181**, 153 (1949).

** The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; D-3 PGA, D-3-phosphoglyceric acid; D-2 PGA, D-2-phosphoglyceric acid; DL-2 PGA, DL-2-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; coenzyme, 2,3-PGA; EDTA, ethylenediaminetetraacetic acid; Tris, tris (hydroxymethyl)-aminomethane; IAA, iodoacetic acid; PCMB, *p*-chloromercuribenzoate.

3) V. W. Rodwell, J. C. Towne and S. Grisolia, *Biochim. et Biophys. acta*, **20**, 394 (1956).

4) V. W. Rodwell, J. C. Towne and S. Grisolia, *J. Biol. Chem.*, **228**, 874 (1957).

5) H. Edelhoch, V. W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

6) L. I. Pizer and C. E. Ballon, *J. Am. Chem. Soc.*, **79**, 3612 (1957).

glycerophosphate according to the method of Kiessling[7] and purified as described by Warburg and Christian[8]. 2,3-PGA was isolated from pig erythrocyte according to the procedure of Greenwald[9]. D-3PGA was obtained from the Nutritional Biochemicals Corporation. Phosphoryl enolpyruvate was synthesized according to the method of Baer and Fischer[10].

Enzyme assay. Determination of the enzyme activity was carried out according to a modification of the polarimetric method of Sutherland et al.[2]. The polarimetric measurement of the PGA mutase activity is based on the change in optical rotation when D-3PGA (or D-2PGA) is converted to D-2PGA (or D-3PGA). In the neutral solution containing molybdate ions, the $[\alpha]_D$ of D-3PGA is $-745°$, whereas that of D-2PGA is $-68°$. When the amount of the substrate (e.g. D-3PGA) which is converted during incubation, is represented as the x fraction (per cent), the following relation is obtained. Similar relations are also obtained when D-2PGA or DL-2PGA is used as the substrate (Table I).

TABLE I. BASAL RELATION IN ENZYME ASSAY

Substrate	(α)
D-3 PGA	$-745+677x$
D-2 PGA	$-68\ -677x$
DL-2 PGA	$-677x$

(α) represented optical rotation of the mixture of 2- and 3-acids. x represented the fraction of the substrate converted (per cent).

The assay systems contained the following in a final volume of 5 ml: 2.5 ml of 6.6×10^{-2}M DL-2PGA (165 μ moles), adjusted to pH 5.9; 0.5 ml of 10^{-3}M 2,3-PGA (0.5 μ mole), adjusted to pH 5.9; 1.5 ml of 10^{-1}M sodium acetate buffer (pH 5.9); and 0.5 ml of the enzyme solution containing 1 to 16 μg of the crystalline protein. The final concentrations of 2,3-PGA and of active D-2PGA were 10^{-4} and 1.65×10^{-2}M, respectively. The reaction was started by the addition of the enzyme solution and the mixture was incubated at 25°C for ten minutes. After incubation, the reaction was stopped by the addition of 2.5 ml of 20 per cent trichloroacetic acid, and the resulting solution was adjusted to pH 6.8 with 2 N sodium hydroxide. To the neutralized solution, 6 ml of 25 per cent ammonium molybdate was added and the final volume was made up with distilled water to 15 ml. Optical rotation was determined with a photoelectric polarimeter attached to a Beckman spectrophotometer model DU using a 1-dm tube. From the changes in optical rotation, the amounts of D-2 PGA converted (or D-3 PGA formed) in this enzyme reaction were calculated. Under the conditions of the standard assay, 1 enzyme unit was defined as that amount which catalyzed the formation of 1 μ mole of D-3 PGA from D-2 PGA. Specific activity was expressed in units per mg of protein. Because the extinction coefficient of 1 per cent solution of the crystalline enzyme in a 1-cm cell at 280 mμ was found to be 13.8 as described later, the protein content of the solution was calculated from the optical density of the enzyme solution at 280 mμ. The reaction rate was proportional to the amount of the enzyme up to 16 μg under the conditions of the standard assay (Fig. 1).

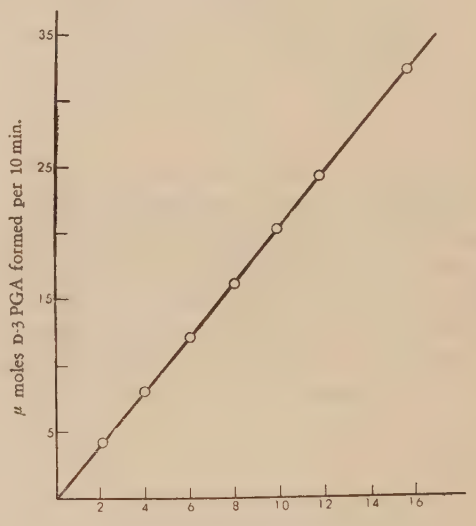
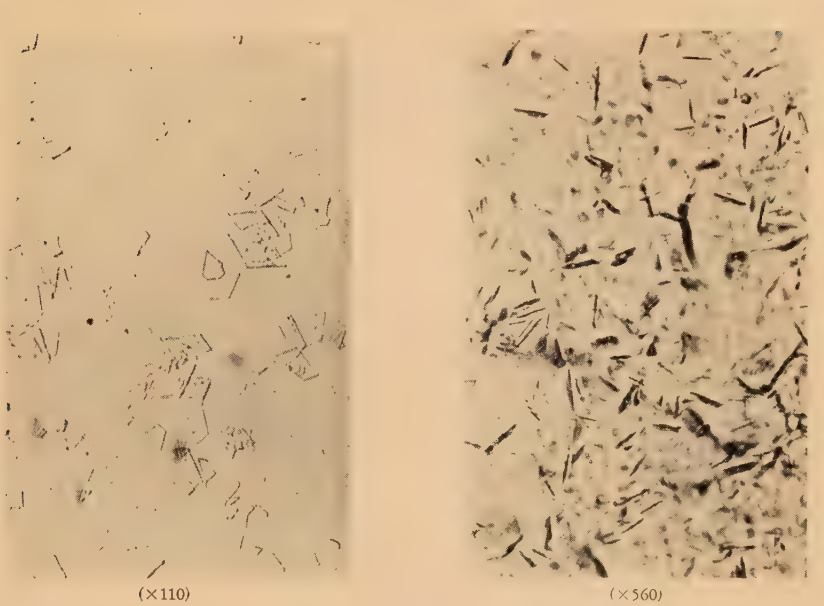


FIG. 1. Proportionality between Rate of Enzyme Reaction and Amount of Enzyme added.
The conditions of reaction were described in the text. The amounts of enzyme as indicated were used.

Preparation of crystalline PGA mutase. The crystalline enzyme was easily prepared by a modification of the procedure of Rodwell et al.[3]. The procedure was as follows. Fragmented, fresh baker's yeast was mixed with one-eighth volume of toluene at 38°C. The liquefied mixture was left for ten hours at 38°C, then cooled to 0°C and diluted with an equal volume of cold distilled water. The solids were removed by centrifugation (30,000 × g, 30 minutes, at 0°C). The supernatant solution was adjusted to pH 7.0 with sodium hydroxide, and then treated with 1 volume of saturated ammonium sulfate solution (pH 7.0) which had been

7) W. Kiessling, *Ber. chem. Ges.*, **68**, 243 (1935).
8) O. Warburg and W. Christian, *Biochem. Z.*, **310**, 384 (1942).
9) I. Greenwald, *J. Biol. Chem.*, **63**, 339 (1925).
10) E. Baer and L. Fischer, *J. Biol. Chem.*, **180**, 145 (1945).



(×110) (×560)

FIG. 2. Recrystallized PGA Mutase.

heated. The suspension was maintained at 75°C for twenty minutes. It was cooled to 0°C and the denatured protein was removed by filtration. The filtrate was then brought to 0.75 saturation with the further addition of saturated ammonium sulfate solution and centrifuged (30,000 × g, 1 hour, at 0°C). The precipitate was dissolved in cold distilled water and diluted with water to bring the protein content to 10 mg per ml. To the 1 per cent solution was added 0.9 volume of acetone, previously chilled to −20°C. The precipitate was collected by centrifugation at −5°C and from this precipitate the enzyme was extracted twice with an adequate volume of cold distilled water. To the extracted solution, saturated ammonium sulfate solution was added to give 0.75 saturation. The precipitate was collected by centrifugation (50,000 × g, 15 minutes, at 0°C). The precipitate was dissolved in a small quantity of distilled water. The clear solution was treated with saturated ammonium sulfate solution added drop by drop until the first appearance of permanent opalescence, and allowed to stand in the ice box. Crystallization of the enzyme began within a few hours and was allowed to continue for twenty-four hours or more. For recrystallization, the crystals were harvested by centrifugation and treated in the same way as described above. The rhombic plate- and rod-shaped crystals were obtained under a slight variation of the conditions during crystallization (Fig. 2).

RESULTS AND DISCUSSION

The crystalline enzyme was usually kept as a suspension in ammonium sulfate mother liquor at 0°C. Under these conditions, its ac-

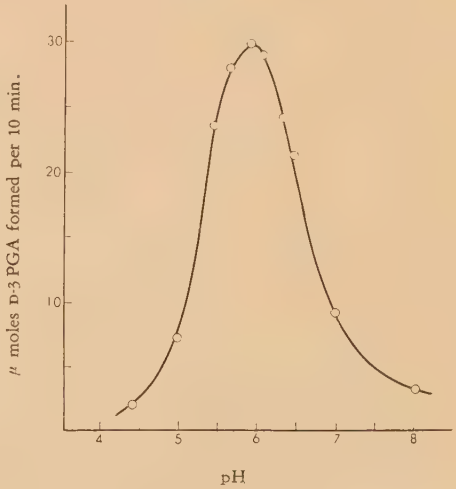


FIG. 3. pH-Activity Curve.

The conditions of reaction were the same as those for the standard assay except that pH values of the reaction mixtures were varied. Fifteen micrograms of the crystalline enzyme were used. In this experiment, below pH 6.0, 0.1 M sodium acetate buffer was used and above pH 6.0, the reaction mixtures were adjusted to required pH with Tris or sodium hydroxide.

tivity remained unchanged for a few months. The absorption spectrum of the crystalline enzyme had a maximum at 280 mμ. The extinction coefficients of 1 per cent solution (determined by dry weight) in a 1 cm-cell at 280 and 260 mμ were 13.8 and 7.0, respectively. The 280 mμ/260 mμ absorption ratio of the enzyme was about 2. The specific activity of the recrystallized enzyme was 2,000.

pH optimum. The effect of pH on the mutase activity was studied over the range of pH 4.5 to 8.0 with DL-2 PGA as the substrate. The pH-activity curve is shown in Fig. 3.

From these results, the pH optimum was 5.9 and this value was found to agree with the result reported by Rodwell et al.[4]

Equilibrium constant. The equilibrium constant (Keq) of the enzyme-catalyzed reaction determined at 30°C by the method using the enolase reaction has been reported to be 6[11] or 6.3[4]. The equilibrium of the enzyme reaction at various pH was investigated with D-3 PGA as the substrate. In this experiment, the residual D-3 PGA was measured in the presence of excess PGA mutase (500 μg) after one hour-incubation at 25°C. When the change in optical rotation was measured, x which represented per cent of the amounts of D-3 PGA converted to D-2 PGA, was calculated from the given equation in Table II. The equilibrium constant at 25°C is given in Table II.

TABLE II. EQUILIBRIUM CONSTANT

PH	α	x (%)	Keq : 3 PGA/2 PGA
5.0	−1.075	16.94	4.90
5.4	−1.088	15.86	5.30
5.9	−1.089	15.75	5.35
6.5	−1.082	16.37	5.10
7.2	−1.086	16.00	5.25

The reaction mixtures contained (in micromoles) D-3 PGA, 137; 2, 3-PGA, 0.25; acetate buffer at various pH as indicated, 150 and enzyme with 500 micrograms of protein. Final volume 5.0 ml. Incubation 1 hour at 25°C. The reaction was terminated by the addition of 2.5 ml of 20 per cent trichloroacetic acid. The precipitate was discarded by filtration and the supernatant solution was neutralized. The amounts of D-3 PGA converted to D-2 PGA were determined polarimetrically. Under the conditions of the polarimetric measurements, the following relation was obtained between α (optical rotation) and x which represented the fraction of D-3 PGA converted (per cent), since D-3 PGA corresponded to 0.1706 per cent solution. $\alpha = 1.155x - 1.271$

From these results, the average value of Keq was 5.2.

Michaelis constant for the coenzyme. Relationship between the rate of the enzyme reaction and the concentration of the coenzyme is shown in Fig. 4. When the concentration of the substrate is constant, the equation represented by Laidler et al.[12] for enzyme reactions which require the coenzyme, is identical with that of Michaelis-Menten[13]. In such a case a double reciprocal manner of Lineweaver and Burk[14] can be used. In this experiment, the final concentration of active D-2 PGA as the substrate was 1.65×10^{-2} M, and 2, 3-PGA was given within the range of 5×10^{-5} to 10^{-3} M.

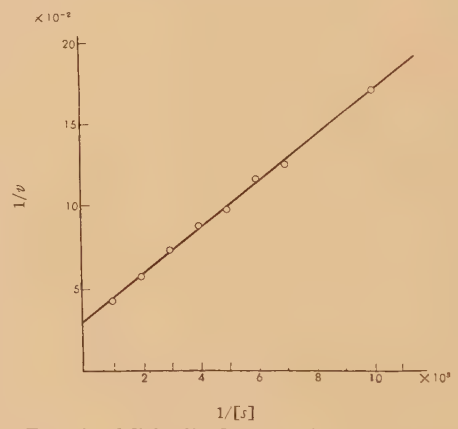


FIG. 4. Michaelis Constant for Coenzyme.

The conditions of reaction were the same as those for the standard assay except that the concentration of 2, 3-PGA was varied. Three micrograms of the crystalline enzyme were used. v is expressed as micromoles of D-3 PGA formed per ten minutes.

From these results, the Michaelies constant for the coenzyme was calculated and found to be 3.31×10^{-4} M. On the other hand, we experienced the fact that the enzyme was inhibited by the substrate, as reported previously[4].

The primary process in the enzyme reaction is the combination of the enzyme protein with

11) O. Meyerhof and P. Oesper, *J. Biol. Chem.*, **179**, 1371 (1949).

12) K. J. Laidler and I. M. Socquet, *J. Phys. Colloid. Chem.*, **54**, 530 (1950).

13) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

14) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

the substrate and the coenzyme in order to form the ternary complex. This is a chemical union among the reactive groups of the substrate and of the coenzyme and the active center of the enzyme protein. It seems that both the substrate and the coenzyme have two reactive groups, phosphate and carboxyl groups. In view of the structural similarity of the coenzyme to the substrate, it may be considered that the enzyme can not apparently distinguish the two compounds. Thence, the coenzyme may compete with the substrate for the active site on the enzyme. Therefore, when the concentration of the substrate is made to increase, the amounts of the coenzyme combined with the enzyme probably decrease and so the activity becomes lower. From such a point of view, it should be noted that the Km value for the coenzyme may be influenced by the substrate concentration.

Effects of compounds, structurally related to the substrate. The effects of the compounds having a similar structure to the substrate on the enzyme reaction were examined. As the final concentration of the substrate, DL-2 PGA, under the tested conditions was 3.3×10^{-2} M, those of such compounds were 10^{-2} M.

TABLE III. EFFECTS OF COMPOUNDS STRUCTURALLY RELATED TO THE SUBSTRATE

Compound	E. U.	Inhibition (%)
Basic System	32.0	—
α-DL-Glycerophosphate	32.0	0
β-Glycerophosphate	29.8	7
DL-Glycerate	32.0	0
DL-Lactate	28.8	10
Phosphoryl enolpyruvate	21.8	32

The conditions of reaction were the same as those for the standard assay except that various compounds tested were given in the final concentration of 10^{-2} M. Sixteen microgsrams of the crystalline enzyme were used.

As shown in Table III, phosporyl enolpyruvate inhibited the activity. It is concluded that only the compound having both phosphate and carboxyl groups may have an affinity towards the active site of the enzyme. It seems that the mode of inhibition caused by phosphoryl enolpyruvate may be competitive inhibition.

Effects of metallic ions and SH-inhibitors. Table IV shows the effects of various metallic ions and SH-inhibitors on the activity.

TABLE IV. EFFECTS OF METALS AND SH-INHIBITORS

Reagent	Final concn. (M)	Inhibition (%)	Reagent	Final concn. (M)	Inhibition (%)
Mn^{++}	10^{-1}	70	Co^{++}	10^{-3}	0
	10^{-2}	10	Fe^{+++}	10^{-3}	0
Mg^{++}	10^{-1}	70	Ag^{+}	10^{-3}	40
	10^{-2}	10	Hg^{++}	10^{-2}	20
Ca^{++}	10^{-1}	70		10^{-3}	0
	10^{-2}	0	Cd^{++}	10^{-2}	30
St^{++}	10^{-1}	57		10^{-3}	0
	10^{-2}	0	Zn^{++}	10^{-3}	67
Ba^{++}	10^{-3}	0		10^{-4}	10
Al^{+++}	10^{-3}	8	Cu^{++}	10^{-2}	72
Cr^{+++}	10^{-3}	7		10^{-3}	20
Ni^{++}	10^{-3}	0	IAA	10^{-2}	7
Pb^{++}	10^{-3}	0	PCMB	3×10^{-5}	6

The conditions of reaction were the same as those for the standard assay with the exception of the addition of various metals and SH-inhibitors. Ten micrograms of the crystalline enzyme were used.

From these results, it is apparent that the tested metallic ions do not activate the enzyme. In relatively high concentrations, Mn^{++}, Mg^{++}, Ca^{++}, Sr^{++}, Ag^{+}, Hg^{++}, Cd^{++}, Zn^{++} and Cu^{++} inhibited the activity. However, as the SH-inhibitors had little effect on this enzyme, metallic ions may not inhibit the activity by the mercaptide formation. If metallic ions (especially Mg^{++}, Mn^{++} and Zn^{++}) form the complex with DL-2 PGA as suggested by Rodwell et al.[4] from the findings of Malmström[15], the substrate concentration is made to decrease. Consequently, the reaction rate may become slow. But it may be probable that the metallic ions combine with the enzyme by chelation and inhibit the enzyme. On the other hand, though these metals did not have a stimulating effect, it is necessary to consider that other metallic ions not tested in this experiment may be effective for activation of the enzyme or that, if the enzyme contains the metal, the

15) B. G. Malmström, *Arch. Biochem. and Biophys.*, **49**, 335 (1954).

enzyme may not require the addition of metallic ions.

Effects of chelating agents. The effects of the chelating agents on the activity are shown in Table V.

In relatively high concentrations, the chelating agents inhibited the enzyme. These results suggest that this enzyme may be a metal enzyme. To elucidate this problem, the enzyme solution was dialyzed against 0.1 M phosphate buffer of pH 7.0 containing 0.1M chelating agents, and then redialyzed against the same buffer containing no chelating agent in order to remove the above reagents from the dialyzed solution. The activity of the redialyed enzyme was recovered completely. From these results, if the enzyme is a metal enzyme, the combination between the metal and the enzyme may be firm.

TABLE V. EFFECTS OF CHELATING AGENTS

Reagent	Final concn.	E. U.	Inhibition (%)
	M		
Basic System	—	32.0	—
EDTA	5×10^{-2}	29.8	7.0
Citrate	//	17.9	44.0
Oxalate	//	11.2	65.0
Pyrophosphate	6×10^{-2}	7.4	77.0
Na azide	10^{-1}	26.4	17.5

The conditions of reaction were the same as those for the standard assay except that various chelating agents were given in the final concentration as indicated. Sixteen micrograms of the crystalline enzyme were used.

Effect of fluoride. Fluoride showed special inhibition on PGA mutase. The relationship between the degree of inhibition and the concentration of fluoride is given in Table VI.

In this experiment, the effect of fluoride was changed by the presence of the chelating agents. When the concentration of EDTA or citrate was higher than that of fluoride, fluoride inhibition was diminished. In these cases, the concentration of EDTA or citrate was that at which the enzyme activity was not inhibited by both compounds. However, when the above chelating agents were given in the lower concentrations than fluoride, these compounds could not prevent fluoride inhibition.

TABLE VI. EFFECT OF FLUORIDE

NaF	EDTA	Citrate	Inhibition (%)
M	M	M	
2×10^{-4}	—	—	0
5×10^{-4}	—	—	15
	—	—	75
10^{-3}	10^{-2}	—	0
	2×10^{-3}	—	15
	10^{-3}	—	39
	2×10^{-4}	—	100
	—	10^{-2}	13
5×10^{-3}	—	—	100
	5×10^{-2}	—	20
10^{-2}	—	—	100
	10^{-2}	—	86

The conditions of reaction were the same as those for the standard assay except other additions as indicated. Ten micrograms of the crystalline enzyme were used.

In the cases of enolase[8] and phosphoglucomutase[16], magnesium fluorophosphate complexes inhibited the enzyme activities. Therefore, in order to elucidate the mechanism of fluoride inhibition, the participation of the metallic ion must be considered. As the chelating agents could prevent fluoride inhibition, there seems to be a contribution of the metallic ion to fluoride inhibition. However, it can not be thought that such a magnesium fluorophosphate complex is formed in the reaction mixture and inhibits the activity, since magnesium ion was not added.

In order to elucidate these problems, further studies are being continued.

SUMMARY

PGA mutase was crystallized in two forms. The activity of the crystalline enzyme was directly assayed by the polarimetric measurement. The optimum pH, the equilibrium constant at 25°C, and the Michaelis constant for the coenzyme were determined.

Among the compounds structurally related to the substrate, only phosphoryl enolpyruvate inhibited the enzyme. In relatively high concentrations various metallic ions showed inhibitory

16) V. A. Najjar, *J. Biol. Chem.*, **175**, 281 (1948).

effects, but the SH-inhibitors had little effect. Moreover the chelating agents given in relatively high concentrations inhibited the enzyme. It is then discussed whether the mutase may be the metal enzyme or not. Fluoride inhibited the activity. However, this inhibition was prevented by the addition of EDTA or citrate, whose concentration was higher than that of fluoride. The mechanism of fluoride inhibition is discussed.

Acknowledgements. The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice and suggestions throughout the course of this work. Thanks are also due to Mr. F. Ibuki for his cooperation in carrying out this experiment.

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Studies on Crystalline Yeast Phosphoglyceric Acid Mutase

II. Electrophoretic Separation of Each Component*

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Received September 9, 1958

Crystalline phosphoglyceric acid mutase isolated from baker's yeast was found to be composed of six components by means of electrophoretic analysis. Each component was completely separated by zone electrophoresis. Among the six components, five of them were PGA mutase** and had different specific activities. Each component revealed a low activity even without the addition of the coenzyme and was inhibited by fluoride in the same manner as in the case of the crystalline mutase.

INTRODUCTION

In the recent paper, Edelhoch et al.[1] have reported that the crystalline yeast PGA mutase behaves as a homogeneous protein by sedimentation analysis, but is not homogeneous electrophoretically.

We have also confirmed that the crystalline enzyme migrates as a heterogeneous protein by moving boundary electrophoresis. From such a result that the crystalline enzyme consists of six components, it seems necessary to decide which component is the PGA mutase protein. At this time, we have succeeded in complete separation of each component by means of zone electrophoresis and have obtained knowledge on each one.

MATERIALS AND METHODS

Crystalline PGA mutase and other materials have been prepared by methods described in the preceding paper[2]. The conditions of the standard assay for the activity have been described[2].

Boundary electrophoretic analysis was performed at 0.7°C with a Tiselius apparatus equipped with a diagonal slit device of Svensson[3]. Mobilities were calculated

* Presented at the Meeting of the Agricultural Chemical Society of Japan, Kyoto, May 3, 1958.

** The following abbreviations are used in this paper: PGA mutase, phosphoglyceric acid mutase; DL-2 PGA, DL-2-phosphoglyceric acid; 2,3-PGA, D-2,3-diphosphoglyceric acid; EDTA, ethylenediaminetetra acetic acid; coenzyme, 2,3-PGA.

1) H. Edelhoch, V. W. Rodwell and S. Grisolia, *J. Biol. Chem.*, **228**, 891 (1957).

2) H. Chiba and E. Sugimoto, This Bulletin, **23**, 207 (1959)

3) H. Svensson, *Kolloid. Z.*, **87**, 181 (1939).

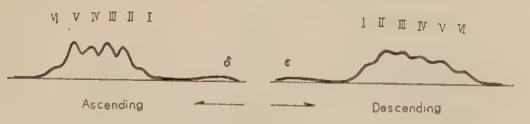
for the descending boundaries from conductivity values determined at 0.7°C.

Preparative zone electrophoresis was carried out according to the method of Bernfeld and Nisselbaum[4]. The supporting medium contained the following: amylose 1.5 per cent; corn starch 3 per cent; hyflo Super Cel 3 per cent; and phosphate buffer of pH 7.0 and 0.07 ionic strength. Amylose was prepared according to the method of Schoch et al[5,6]. To make a stiff starch gel as a supporting medium, the mixed paste described above was boiled and poured into the rectangular electrophoresis cell made of glass plates (30×1 cm base, 1 cm high), and was allowed to cool overnight. The starch paste hardened to a stiff gel. The slot (0.2×0.75 cm base, 0.9 cm high) filled with the enzyme solution, was made in the gel, about 4 cm inside from one side. During zone electrophoresis, the electrophoretic cell was covered with vinyl sheets in order to avoid evaporation.

RESULTS AND DISCUSSION

Boundary electrophoresis. Prior to moving boundary electrophoresis, crystalline PGA mutase was dissolved in a phosphate buffer of pH 7.0 and 0.1 ionic strength to a concentration of approximately 4 per cent protein solution, and dialyzed against the same buffer for two days in the ice box. Electropohoretic analysis was carried out in the same buffer at a potential gradient of 4.6 volts per cm for 10,800 seconds (0.7°C). The pattern is shown in Fig. 1.

From these results the crystalline enzyme was



Mobility

I	II	III	IV	V	VI
2.64	3.13	3.68	4.28	4.87	5.65

FIG. 1. Boundary Electrophoretic Pattern of Crystalline PGA Mutase.

The pattern was obtained from 3 per cent protein solution in a phosphate buffer of pH 7.0 and 0.1 ionic strength at a potential gradient of 4.6 volts per cm for 10,800 seconds. The numbers refered to the component. Mobilities were negative in sign and were expressed as cm^2 per volt per second $\times 10^{-5}$.

found to be a heterogeneous system consisting of six components. This fact is similar to the result that the enzyme preparation crystallized by Rodwell et al.[7] had five components.[1]

On the other hand, the crystalline enzyme contains a small amounts of organic phosphorus[8]. Therefore, if enzyme-bound phosphate is not uniformly distributed on the enzyme as pointed out by Edelhoch et al.[1], the enzyme may behave as a heterogeneous protein, since the difference in net charge of each component produces various ionic forms of the enzyme protein. But if the crystalline preparation is not pure, it is necessary to decide which component is the true PGA mutase. In order to separate each component completely, preparative zone electrophoresis was employed.

Zone electrophoresis. The crystalline enzyme was dissolved in a phosphate buffer of pH 7.0 and 0.07 ionic strength, and dialyzed against the same buffer before horizontal zone electrophoresis was performed. The slot was filled with 0.12 ml of the dialyzed enzyme solution containing 3.2 mg of protein. A potential difference of 180 volts between the two electrodes was then applied for twenty five hours.

In order to observe the distribution of the separated components in the gel, the thin slice was cut off from the surface of the gel by sliding the cutting blade parallel to the bottom of the gel. The slice thus obtained was transferred to a dish for staining the cut surfaces. The protein detecting dye, Amido Black 10 B which was saturated in methanol-distilled water-glacial acetic acid (50 : 50 : 10, v/v), was then poured over them. The positions of the separated components are shown in Fig. 2-A. From this result, it was evident that six components were completely separated one another by means of zone electrophoresis.

In order to collect the separated components, the gel was cut transversely into 0.5 cm sections. Each section was eluted with 2 ml of 0.1 M

4) P. Bernfeld and J. S. Nisselbaum, *J. Biol. Chem.*, **220**, 851 (1956).
5) T. J. Schoch, *J. Am. Chem. Soc.*, **64**, 2954 (1942).
6) E. J. Wilson, Jr., T. J. Schoch and C. S. Hudson, *J. Am. Chem. Soc.*, **65**, 1380 (1943).
7) V. W. Rodwell, J. C. Towne and S. Grisolia, *Biochim. et Biophys. acta*, **20**, 394 (1956).
8) V. W. Rodwell, J. C. Towne and S. Grisolia, *J. Biol. Chem.* **228**, 875 (1957).

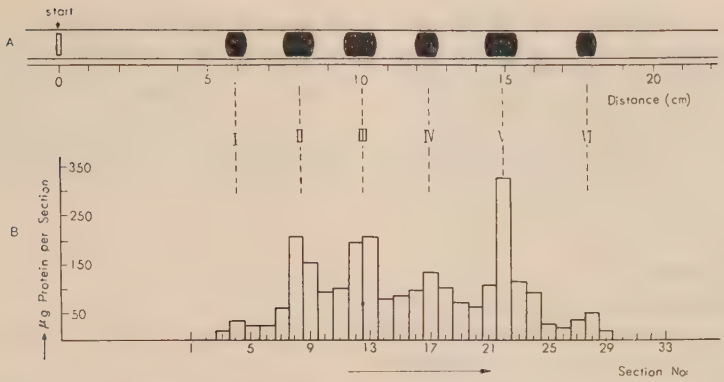


FIG. 2. Separation of Individual Components by Zone Electrophoresis.

A: An illustration of each position of separated components on the starch gel detected by staining as described in the text.
B: The pattern of each protein content of separated components.

Zone electrophoresis conditions: phosphate buffer, pH 7.0, ionic strength 0.07; 180 volts potential difference between electrodes (21 mA); amount of protein, 3.2 mg; duration 25 hours. Eluate from each section was about 4.2 ml. The arrow indicated the position of the starting zone.

acetate buffer of pH 5.9 with a Potter-Elvehjem homogenizer, and the mixture was centrifuged. Such elution was repeated twice with a 1 ml portion of the same buffer. The protein content of each section was determined by means of the method of Lowry et al.[9]. From the relation between the optical density at 280 mμ and the Lowry color intensity of the crystalline enzyme, the protein content (dry weight) was calculated. The correction value derived from the starch mixture itself was subtracted from the enzyme protein content found in each section. The recovery of the applied enzyme protein was approximately 100 per cent. When the protein content per section was plotted against the section number, the pattern was obtained (Fig. 2-B).

This pattern is analogous to that obtained by the method of moving boundary electrophoresis. Hereupon, the complete separation of each component has been established.

Confirmation of PGA mutase activity in components. To examine each component as to the PGA mutase activity, the following sections were chosen by reason that they represented each pure component without contamination of the other: No. 4 section for component I; No. 8 for component II; No. 12 for component III; No. 17 for component IV; No. 22 for component V; and No. 28 for component VI. The assay of the enzyme activity was carried out under the standard condition. The specific activities of these components are given in Table I.

TABLE I. SPECIFIC ACTIVITIES OF INDIVIDUAL COMPONENTS

Component	Enzyme μg	E. U.	S. A.
I	3.7	19.5	5,280
II	7.4	29.0	3,920
III	7.4	19.6	2,656
IV	14.8	20.4	1,376
V	74.0	5.6	75
VI	14.8	0	0
Original	14.8	29.6	2,000

The conditions of reaction were the same as those for the standard assay[2]. The amounts of enzyme as indicated were used.

Among the six components, five of them (I–V) had the PGA mutase activity, whereas component VI had no activity. The specific activity of component I was the highest and the others became lower in order.

From the fact that I–V components were PGA mutase, even though specific activities of individual components were not identical, it may be concluded that the proteins of individual

9) O. H. Lowry, N. J. Rosebrough, A. L. Far and R. J. Randal, *J. Biol. Chem.*, **193**, 265 (1951).

components are not different essentially, but that those proteins have only dissimilar net charges caused by the amounts of bound phosphate, as discussed in the previous section.

But the fact that the slower migrating component had a higher specific activity than the faster one shows that there is an inverse relationship between the specific activity and the amounts of bound phosphate.

Coenzyme requirement of each component. In order to investigate a coenzyme requirement of each component, the activities were examined without the addition of the coenzyme.

TABLE II. ACTIVITIES OF INDIVIDUAL COMPONENTS IN THE ABSENCE OF THE COENZYME ADDED

Component	Enzyme	D-3 PGA formed	
	μg	μ moles	μ moles/10 min./mg of protein
I	7.4	16.9	570
II	7.4	13.4	452
III	14.8	17.0	287
IV	29.6	15.1	127
V	74.0	5.0	17

The conditions of reaction were the same as those for the standard assay except that the coenzyme was omitted. The reaction mixture was incubated for forty minutes. The amounts of individual components as indicated were used.

As evident from Table II, components I–V had small activities even in the absence of the added coenzyme. But the activities under the above mentioned conditions decreased to about one-tenth of those under the conditions of the standard assay. Therefore, though each component requires the addition of the coenzyme in order to reveal its maximum activity, it is probable that small amounts of the coenzyme combine with each component of the enzyme. Moreover, it seems that the component having a higher specific activity contains a larger amount of the bound coenzyme than that having a lower specific activity, because, even in the case of no addition of the coenzyme, the former showed a higher activity than the latter.

Effect of fluoride on each component. As previously demonstrated fluoride shows a marked inhibitory action against the mutase activity. In the case of each component fluoride acted in the same manner as in the case of the crystalline enzyme (Table III).

TABLE III. EFFECT OF FLUORIDE ON ACTIVITIES OF INDIVIDUAL COMPONENTS

Component	NaF	EDTA	Inhibition (%)
	M	M	
I	10^{-3}	—	72
	〃	10^{-2}	0
II	〃	—	74
	〃	10^{-2}	0
III	〃	—	73
	〃	10^{-2}	0
IV	〃	—	70
	〃	10^{-2}	0
V	〃	—	68
	〃	10^{-2}	0

The conditions of reaction were the same as those described in Table I with the exception of other additions as indicated.

Each component was also strongly inhibited by fluoride at the concentration of 10^{-3} M and fluoride inhibition was prevented by the addition of the chelating agent (EDTA), which had been given in higher concentrations than fluoride.

As described in this paper, PGA mutase has an attractive character judging from the fact that the enzyme consists of five components having various specific activities. Therefore further studies on each component seem to be necessary in order to elucidate the mechanism of the mutase reaction.

SUMMARY

Crystalline PGA mutase was recognized as a heterogeneous protein by moving boundary electrophoresis and was then completely separated into each component by zone electrophoresis.

Among the six components obtained, five of them (I–V) had the PGA mutase activity, and the specific activity of component I was the highest and the others became lower in order. The differences among the properties of those components were also discussed.

As the components (I–V) had a small activity in the absence of the added coenzyme, it is considered that a small amount of the coenzyme combines with each component.

Though fluoride inhibited the activity of each component, it could not inhibit its activity in the presence of chelating agents.

Acknowledgements. The authors wish to express their thanks to Prof. H. Mitsuda of Kyoto University for his kind advice and encouragement throughout the course of this work. The authors are also grateful to Mr. K. Hasegawa for his technical assistance in carrying out zone electrophoresis.

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The Chemistry of Ilexol. IV.*

The Identity of Acetate A with Urs-13 (18)-en-3β-yl Acetate together with the Formation of Isoilexone, Isoilexenedione and Isoilexene

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It has now been shown that Acetate A[1] previously obtained by isomerizing ilexol acetate is nothing other than urs-13 (18)-en-3β-yl acetate prepared[2] from α-amyrin, furnishing conclusive evidence for its conversion into one of the compounds of the ursane series.

Isoilexol, oxidized with chromium trioxide at room temperature, afforded a ketone named isoilexone, $C_{30}H_{48}O$, m.p. 194–195°, $[\alpha]_D^{25}+75.90°$ (c, 0.527), and oxidized with the same oxidant at 70–80°, another ketone named isoilexenedione, $C_{30}H_{46}O_2$, m.p. 221–223°, $[\alpha]_D^{28}+16.28°$ (c, 0.307).

Huang-Milon reduction of these ketones afforded one and the same deoxy compound named isoilexene, $C_{30}H_{50}$, m.p. 183–185°, $[\alpha]_D^{27}+48.34°$ (c, 0.290), which might well be assumed to constitute a pair of optical antipodes with olean-13 (18)-ene, $C_{30}H_{50}$, m.p. 184–185°, $[\alpha]_D^{25}-48.50°$ (c, 0.545).

In previous papers of this series[1], it has been shown that Acetate A (VIII), $C_{32}H_{52}O_2$, m.p. 212–213°, $[\alpha]_D^{19}$ −22.70° (c, 0.488), a compound previously obtained by isomerizing ilexol acetate through three different pathways, might be identical with urs-13 (18)-en-3β-yl acetate (X) because of good agreement in their physical constants between both substances.

By comparison of their m.ps., infrared spectra (Fig. I, f–g) and optical rotations, Acetate A (VIII) has been shown to be identical with urs-13 (18)-en-3β-yl acetate (X) prepared according to the precedure of previous groups of workers[2,3], furnishing conclusive evidence for its conversion into a compound of the ursane series.

* Part III, This Bulletin, **22**, 131 (1958). Presented at the General Meeting of the West Japan Branch of the Agricultural Chemical Society of Japan held July 5, 1958, at Kyushu University, Fukuoka, Japan.

** Ooemachi, Kumamoto City, Japan.

1) K. Yagishita, This Bulletin, **22**, 123, 131 (1958).

2) J. D. Easton, W. Manson and F. S. Spring, *J. Chem. Soc.*, **1953**, 943.

3) J. M. Beaton, F. S. Spring, R. Stevenson and W. S. Strachan *ibid.*, **1955**, 2610.

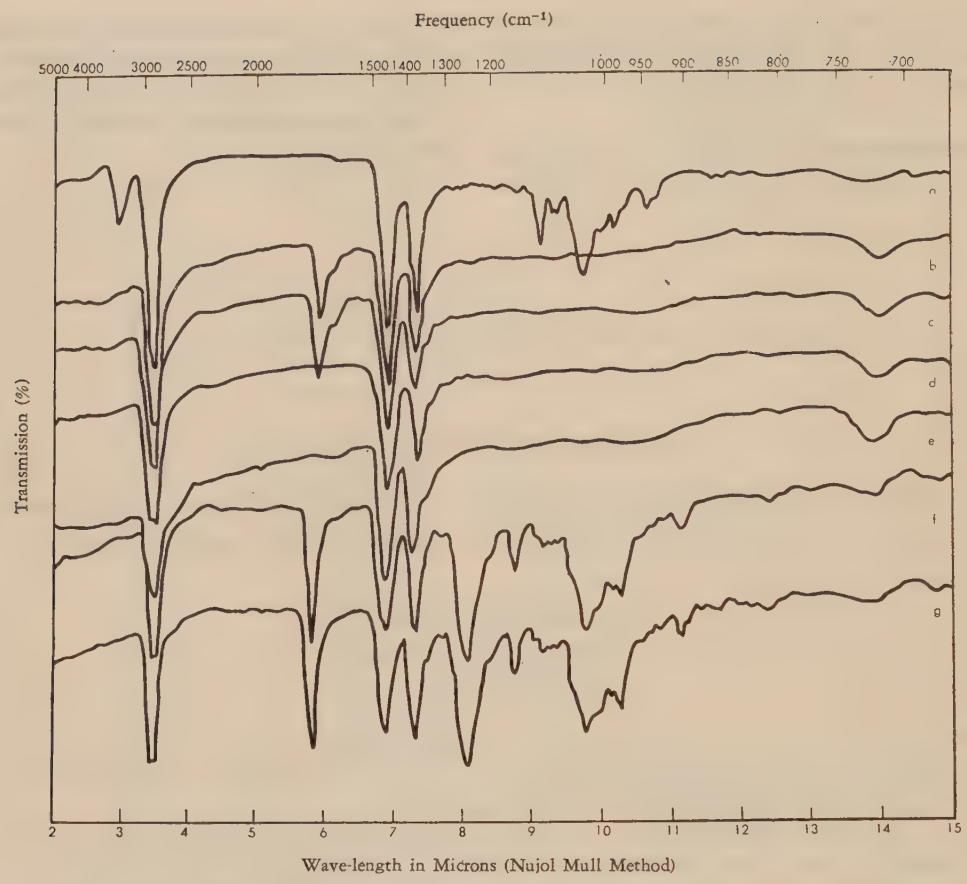

FIG. 1. a): Isoilexol, b): Isoilexone, c): Isoilexenedione, d): Isoilexene, e): Olean-13 (18)-ene, f): Acetate A, g): Urs-13 (18)-en-3β-yl Acetate.

Isoilexol (V), an isomer of ilexol obtained by isomerizing ilexol, ilexol acetate and ilexene, was oxidized with chromium trioxide at room temperature to a ketone named isoilexone (XI), $C_{30}H_{48}O$, m.p. 194–195°, $[\alpha]_D^{25}$+75.90° (c, 0.527).

However, (V), oxidized with the same oxidant at 70–80°, afforded another ketone named isoilexenedione (XII), $C_{30}H_{46}O_2$, m.p. 221–223°, $[\alpha]_D^{28}$+16.28° (c, 0.307).

It was found that reduction with sodium and ethanol of both isoilexone and isolexenedione regenerates the original isoilexol, whereas, Huang-Minlon reduction of (XI) and (XII) afforded one and the same deoxy compound named isoilexene* (XIII), $C_{30}H_{50}$, m.p. 183–185°, $[\alpha]_D^{27}$ +48.34° (c, 0.290).

Except for its dextrorotation, isoilexene (XIII) agreed, in every respect, with olean-13 (18)-ene (XVIII), $C_{30}H_{50}$, m.p. 183–185°, $[\alpha]_D^{25}$ +48.50° (c, 0.545) prepared according to the procedure of previous workers[4], indicating that both substances might well constitute a pair of

* In analogy with this result, the rearrangement of ursa-9 (11): 12-dien-3β-yl acetate into oleana-11 : 13 (18)-dien-3β-yl acetate has been reported by J. M. Beaton, F. S. Spring, R. Stevenson, and R. Strachan[3], and by J. I. Shaw, F. S. Spring, and R. Stevenson (*J. Chem. Soc.*, **1956**, 465); and that of ursa-11 : 13 (18)-dien-3β-yl acetate into oleana-11 : 13 (18)-dien-3β-yl acetate by J. M. Beaton et al.[3].

4) G. Brownlie, M. B. E. Fayez, F. S. Spring, R. Stevenson and W. S. Strachan, *J. Chem. Soc.*, **1956**, 1377.

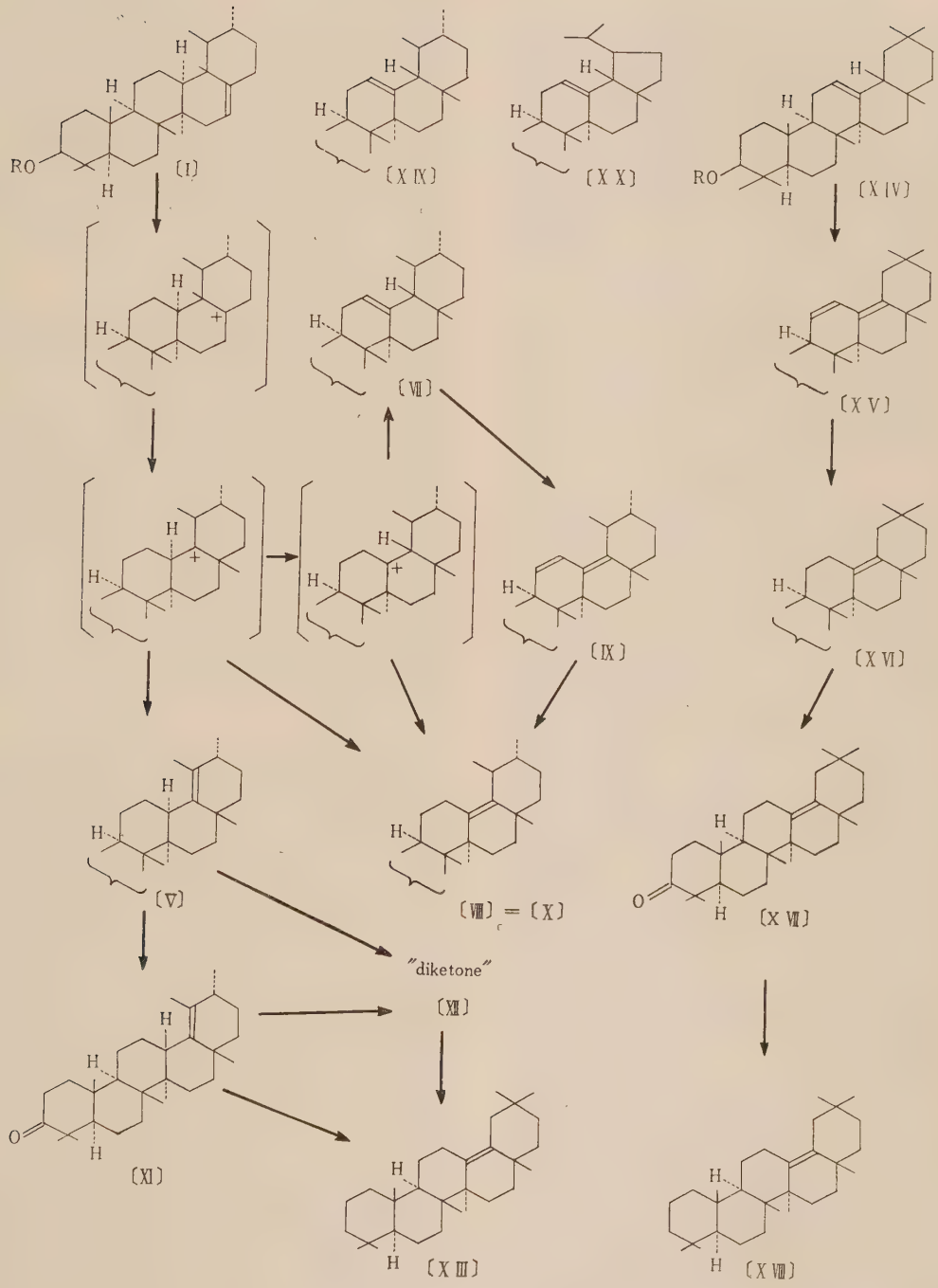
RO
〔I〕
〔XIX〕
〔XX〕
RO
〔XIV〕
〔VII〕
〔XV〕
〔IX〕
〔XVI〕
〔V〕
〔VIII〕 = 〔X〕
O
〔XVII〕
"diketone"
〔XII〕
O
〔XI〕
〔XIII〕
〔XVIII〕

Chart I

optical antipodes, one another.

On the basis of (a) a detailed examination of the infrared spectra of the ursane derivatives[5], (b) a nuclear magnetic resonance study[6], and (c) an unambiguous synthesis of α-amyrin from glycyrrhetic acid of the β-amyrin series[7], the structure with a six-membered ring E (XIX) has recently been ascribed to α-amyrin in place of the alternative structure with a five-membered ring E (XX)[8].

Finally, it should be noted that the foregoing result of experiments accounts for, without any discrepancy, the validity of the structure with a six-membered dring E (I) forwared by the author for ilexol[1].

Experimental*

Preparation of Ursa-11 : 13(18)-dien-3β-yl Acetate (IX). (a) The compound was prepared in essentially the same manner as described by J. D. Easton et al.[2]. A suspension of 8.0 g of α-amyrin acetate and 12 g of selenium dioxide in 150 ml of dioxane was heated in a sealed tube on an oil-bath at 200° for 20 hrs. After being cooled, the resulting reaction mixture was filtered, diluted with an amount of water, and taken up in ether. The ethereal solution was thoroughly washed with water, and dried with Na_2SO_4. Removal of ether gave the residue (7.7 g), which was dissolved in petroleum ether (300 ml), and chromatographed on a column of alumina (15 × 2 cm). Elution with the same solvent (800 ml) and subsequent removal of the solvent afforded a crystalline mass, which, after several crystallizations from chloroform-methanol (1 : 1), crystallized as colorless plates (6.5 g), and had m.p. and mixed m.p. 223–224° either alone or on admixture with the authentic specimen of α-amyrin acetate.

Subsequent elution with petroleum ether-benzene (10:1) and removal of the solvent mixture afforded 320 mg of the crystalline residue, m.p. 185–198°, which was found to be a mixture, and hence, discarded. Further elution with the same solvent mixture (1200 ml) afforded the eluate, which, after removal of the solvent mixture and several crystallizations from chloroform-methanol (1 : 1), crystallized as colorless needles (150 mg), and had m.p. 204–205°, and $[\alpha]_D^{25}$ −76.55° (c, 0.452). M. ps. 206–207°,[2] 204–206°,[3] $[\alpha]_D$ −77°, −79° (c, 1.2, 0.35, $CHCl_3$)[2], −76° (c, 1.0, $CHCl_3$)[3] were recorded for ursa-11 : 13(18)-dien-3β-yl acetate.

Anal. Found: C, 82.51; H, 10.99. Calcd. for $C_{32}H_{52}O_2$: C, 82.34; H, 10.80.

It gave a reddish-brown coloration with tetranitromethane.

(b) The compound (IX) was also prepared as described below. A suspension of 8.0 g of α-amyrin acetate and 12 g of selenium dioxide in 150 ml of amyl alcohol was refluxed on an oil-bath for 40 hrs. After the reaction was over, the filtrate was taken to dryness *in vacuo*, and the crystalline residue was taken up in ether. After washing with water and drying, removal of ether afforded a reddish-brown crystalline mass, which was dissolved in petroleum ether, and purified in just the same manner as described above. The pure substance had m.p. and mixed m.p. 204–205° on admixture with the foregoing specimen. The yield was found to be rather poor (about 80 mg).

Hydrogenation with Platinum-catalyzed Hydrogen of Ursa-11:13(18)-dien-3β-yl Acetate (IX)-Formation of Urs-13(18)-en-3β-yl Acetate (X). An amount of 200 mg of (IX) in 90 ml of ethyl acetate-glacial acetic acid (4 : 5) was shaken with 200 mg of prereduced Adams' catalyst in an atmosphere of hydrogen at room temperature for 24 hrs. After filtration and removal of the solvent mixture, the crude hydrogenation product was deacetylated with 3% ethanolic KOH-solution, giving an alcohol, which, after several crystallizations from ethanol, crystallized as colorless plates (120 mg), and had m.p. 200–201°, $[\alpha]_D^{25}$ −35.03° (c, 0.290). M. ps. 204–205°,[2] 201–202°;[3] $[\alpha]_D$ −37° (c, 1.2, $CHCl_3$)[2], −37° (c, 0.6, $CHCl_3$)[3] were recorded for urs-13(18)-en-3β-ol.

Anal. Found: C, 84.43; H, 11.99. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

It gave a yellow coloration with tetranitromethane. This substance did not depress the m.p. of Alcohol A[1] (m.p. 202–203°).

Reacetylation with acetic anhydride-pyridine afforded crude acetate, which, crystallized several times from

5) A. R. H. Cole, D. W. Thornton and D. E. White, *Chemistry & Industry*, **1956**, 795.
6) G. G. Allan, *ibid.*, **1958**, 529.
7) E. J. Corey and Z. W. Cantrall, *J. Am. Chem. Soc.*, **80**, 499 (1958).
8) G. G. Allan, J. M. Beaton, J. I. Shaw, F. S. Spring, R. Stevenson, J. L. Stewart and W. S. Strachan, *Chemistry & Industry*, **1955**, 281; G. G. Allan, M. B. E. Fayez, F. S. Spring, and R. Stevenson, *J. Chem. Soc.*, **1956**, 456.

* Unless otherwise stated, (a) all melting points were corrected; (b) rotations were measured in $CHCl_3$ solution, using 1-dm tubes; (c) infrared spectra were measured in Nujol with a Perkin-Elmer Model 21 double-beam instrument equipped with rock-salt prisms; (d) liquid chromatography was carried out using alumina (200-mesh, in size) purchased from Wako Pure Chemicals Co., and petroleum ether (b.p. 60-80°).

chloroform-methanol (1 : 1), crystallized as colorless needles, and had m.p. 212–213°, $[\alpha]_D$ −22.52° (c, 0.405). M. ps. 214–216°,[2] 213–215°;[3] $[\alpha]_D$ −23° (c, 1.4, $CHCl_3$)[2], −22° (c, 1.1, $CHCl_3$)[3] were recorded for urs-13(18)-en-3β-al acetate.

Anal. Found: C, 81.78; H, 11.19. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

It gave a yellow coloration with tetranitromethane. This acetate, also, did not depress the m.p. of Acetate A (m.p. 212–213°), showing the idendity of both acetates.

Oxidation with Chromium Trioxide of Isoilexol (V). (a) Formation of Isoilexone (XI). To a solution of 500 mg of isoilexol in 60 ml of benzene-glacial acetic acid (1 : 5), was added 180 mg of chromium trioxide in 20 ml of glacial acetic acid at room temperature during 1 hr. with vigorous stirring. After being left at room temperature overnight, the resulting reaction mixture was diluted with a sufficient amount of water, and taken up in ether. The ethereal layer was washed with water and dried with Na_2SO_4. Removal of ether afforded the residue, which was dissolved in 50 ml of petroleum ether-benzene (5 : 2), and chromatographed on a column of alumina (6×2 cm). Elution with the same solvent mixture afforded a crystalline mass (430 mg), which, crystallized several times from ethanol, crystallized as colorless needles, and had m.p. 194–195°, $[\alpha]_D^{25}$ +75.90° (c, 0.527).

Anal. Found: C, 84.86; H, 11.42. Calcd. for $C_{30}H_{48}O$: C, 84.84; H, 11.39.

Its solubility in organic solvents was found to be almost the same as that of ilexone. It gave an orange coloration with tetranitromethane. The infrared spectrum is shown in Fig. Ib.

(b) Formation of Isoilexenedione (XII). The foregoing oxidation was carried out at 70–80° on a water-bath for 30 min. After being left overnight, the reaction mixture was worked up in the same manner as described above. Several crystallizations from ethanol afforded isoilexenedione, which crystallized as glistening plates (400 mg), and had m.p. 221–223°, and $[\alpha]_D^{28}$ +16.28° (c, 0.307).

Anal. Found: C, 82.55; H, 11.14. Calcd. for $C_{30}H_{46}O_2$: C, 82.13; H, 10.57.

It gave an orange coloration with tetranitromethane. The infrared spectrum is shown in Fig. Ic.

Further oxidation of isoilexone in the same manner as above, afforded the product, which was readily identified as isoilexenedione.

Ladenburg Reduction of Isoilexone (XI) and Isoilexenedione (XII)—Regeneration of Isoilexone (V). (a) To a solution of 100 mg of isoilexone in 30 ml of absolute ethanol, was added in portions 1 g of sodium during 10 min., and the resulting reaction mixture was further refluxed for 2 hrs. After being processed in the usual manner, the reduction product was dissolved in 50 ml of petroleum ether-benzene (5 : 2), and chromatographed on a column of alumina (6×2 cm). Elution with the same solvent mixture (300 ml) followed by removal of the solvent mixture afforded a crystalline mass, which, crystallized several times from methanol, crystallized as colorless prismatic needles (750 mg), and had m.p. 177–178°, undepressed on admixture with an authentic specimen of isoilexol.

(b) Isoilexenedione (900 mg), reduced in the same manner as above, also regenerated isoilexol (630 mg).

Huang-Minlon Reduction of Isoilexone (XI) and Isoilexenedione (XII)—Formation of Isoilexene (XIII). (a) To a suspension of 120 mg of isoilexone in 50 ml of ethylene glycol was added 3 ml of hydrazine hydrate (80%), and the mixture was refluxed on an oil-bath for 1 hr. After being cooled, 500 mg of KOH was added to the reaction mixture, and the mixture was further refluxed for 8 hrs. After being cooled and diluted with water the resulting reaction mixture was thoroughly extracted with benzene. Removal of benzene afforded the residue, which was dissolved in 100 ml of petroleum eeher, and chromatographed on a column of alumina (6×2 cm). Elution with the same solvent (500 ml) followed by removal of the solvent afforded a crystalline mass (65 mg), which, crystallized several times from chloroform-methanol (1 : 1), crystallized as colorless plates, and had m.p. 183–185°, $[\alpha]_D^{27}$ +48.34° (c, 0.290).

Anal. Found: C, 87.52; H, 12.49. Calcd. for $C_{30}H_{50}$: C, 87.73; H, 12.27.

It gave an orange coloration with tetranitromethane. The infrared spectrum is shown in Fig. Id. It was found to be sparingly soluble in ethanol, and methanol, and readily soluble in acetone, ethyl acetate, chloroform, and petroleum ether.

(b) Isoilexenedione (100 mg), reduced in the same manner as above, afforded one and the same compound, i.e. isoilexene (45 mg).

Preparation of Olean-13(18)-ene (XVIII). Starting with 5.0 g of β-amyrin acetate (XIV), the compound was prepared in essentially the same manner as reported by G. Brownlie et al.[4]. In agreement with their data, pure olean-13(18)-ene crystallized as colorless blades from chloroform-methanol (1 : 1), and m.p. 183–185°, $[\alpha]_D^{25}$

$-48.50°$ (c, 0.545). Yield, 125 mg.

Anal. Found: C, 87.52; H. 12.28. Calcd. for $C_{30}H_{50}$: C, 87.83; H, 12.27.

Its Infrared pattern agreed with that of isoilexene (Fig. Ie).

The mixture of this substance and isoilexene (m.p. 183–185°) melted at 181–182°, and it was found to be optically inactive.

Acknowledgements. The author wishes to express his sincere thanks to Prof. Dr. S. Iseda of this Laboratory, to Prof. Dr. Y. Oshima of Kyushu University, and to Prof. Dr. E. Sebe of Kumamoto University, for their helpful advice and encouragement throughout the course of this work. He is also deeply indebted to Messrs. H. Kozuma and K. Kawasaki of the Technical Department of Minamata Factory, Shin Nippon Chisso Hiryo and Co., Minamata, for their kindness for measuring infrared spectra, and to Miss M. Kinoshita of Kumamoto University, for carrying out elementary analyses.

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Effect of Carbon and Nitrogen Sources on the Utilization of Tyramine and Phenolsulphatase Biosynthesis by Cell Suspensions of *Aerobacter aerogenes*.

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Received September 15, 1958

Cell suspensions of *Aerobacter aerogenes* formed a remarkable amount of phenolsulphatase and dissimilate tyramine, provided a suitable primer was present with tyramine. As a primer, many carbon sources, especially, a mixture of amino acids could be considered useful. Several compounds such as glucose strongly inhibited not only the utilization of tyramine but also the phenolsulphatase synthesis of the cell suspensions containing tyramine and casein acid-hydrolyzate, while other compounds such as xylose either ineffective or only a little effective to both of the reactions. Therefore, the possibility that there might be a functional relationship between the ultilization of tyramine and the phenolsulphatase synthesis of *Aerobacter aerogenes* was considered.

From previous studies[1,2] it has been indicated that tyramine induces the phenolsulphatase (hereafter, referred to as PSase) synthesis of *A. aerogenes* and its specificity as an inducer is very high. Attempts to induce the PSase by some substrates, such as phenyl sulphate, *p*-nitrophenyl sulphate and urine-indican have resulted in failure. It was also found that the effect of tyramine is observed not only in this bacterial species, but also in *Salmonella schottmuelleri*, species of *Aspergillus*, and *Penicillium*[3].

It therefore seems that the biochemical mechanism involved in it is worthy of further study. Such a study would lead us to find the net role of tyramine for the enzyme synthesis.

1) T. Harada and F. Hattori, This Bulletin, **20**, 110 (1956).
2) T. Harada, This Bulletin, **21**, 267 (1957).
3) Unpublished data.

First of all, it is desirable to obtain information concerning the presence or absence of the functional relationship between the utilization of tyramine by the organisms and their PS ase synthesis. Therefore, the effects of several carbon and nitrogen sources on the utilization of tyramine and PS ase biosynthesis of *A. aerogenes* were investigated.

METHODS

A. aerogenes ATCC 9621 was employed. This strain hardly produced PSase unless tyramine was supplied in the medium. The medium which was used to obtain cells, had the following composition: Bacto-casamino acids, 0.5 per cent; KH_2PO_4 and K_2HPO_4, each 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, and NaCl, each 0.001 per cent; pH 7.2. Resting cell suspensions were obtained by growing *A. aerogenes*, strain 9621 ATCC, in this medium at 34°C for 48 hours, using a one per cent inoculumn from a yeast extract broth culture. Harvesting was accomplished by centrifugation, the cells were washed thrice with distilled water and the final cell suspension was adjusted to the concentration of 5 mg dry cell material per one ml with 0.2M phosphate buffer, pH 7.2.

To test the effects of carbon and nitrogen sources on the utilization of tyramine and PSase induction, 4 ml of each test solution containing the compounds to be tested and 10^{-2}M tyramine as an inducer were added into 4 ml of this cell suspension and the mixture aerated on a reciprocal shaker at 34°C. A quantity of 0.5 ml of the mixture was pipetted at appropriate intervals and 3 ml of distilled water was added. Immediately the cells were centrifuged at about 5°C. The supernatant was tested to determine the amount of tyramine as mentioned below. The cells, thus obtained were suspended into one ml of water and this suspension was transferred to a graduated test tube already containing 2 ml of acetate buffer, pH 7.2 and one ml of 0.005M *p*-nitrophenyl sulphate solution which had been preliminary incubated at 34°C for five minutes. The content of the tube was immediately brought to a final volume of 5 ml with distilled water and was incubated further under occational shaking for 10 minutes at 34°C. The amount of *p*-nitrophenol produced by the enzymatic action was estimated according to the method already mentioned[4]. PSase activity was expressed in units of optical density. This was determined by a Kotaki AKA photoelectric colorimeter using a S-43 filter and a cell of 0.5 cm in width.

Two ml of each supernatant obtained from the induction mixture, was pipetted and 0.5 ml of 0.5N sodium hydroxide and 3.5 ml of distilled water were added to this solution. The absorption of these solutions at wavelength of 294 mμ was determined by a spectrophotometer (Hitachi type) in order to find the amount of remained tyramine. Such as alkaline solution of tyramine had a maximal absorption at 294 mμ, while a maximal absorption of a solution of tyramine hydrochloride was found at 275 mμ as seen in Fig. 1.

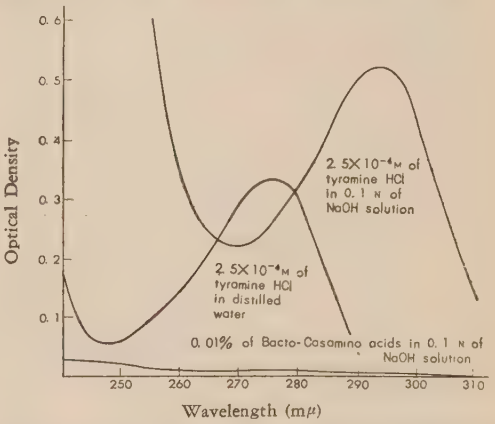

FIG. I. The Absorption Spectra of Tyramine and Bacto-Casamino Acids.

Although an alkaline solution of tyrosine also showed a maximal absorption in the neighborhood of 294 mμ, the amount of tyrosine contained in added 0.2 per cent casein acidhylrolyzate was so small that its absorption was too weak to be recognized in comparison with that of added 5×10^{-3}M tyramine (Fig. 1). Paper chromatographic studies indicated tyramine being utilized as no compound having the construction of phenol seemed to be accumulated in the incubation mixture.

Turbidity of the induction mixture was determined by a Kotaki AKA photoelectric colorimeter using a S-66 filter. As casein acidhydrolyzate Bacto-casamino acids were used.

RESULTS

Effect of Nitrogen Sources on the Utilization of Tyramine and PSase Biosynthesis.

The cell suspensions prepared from the medium without tyramine as described in the experimental method, showed no PSase activity and could

4) T. Harada and K. Kono, *J. Agr. chem. Soc. Japan*, **28**, 608 1954).

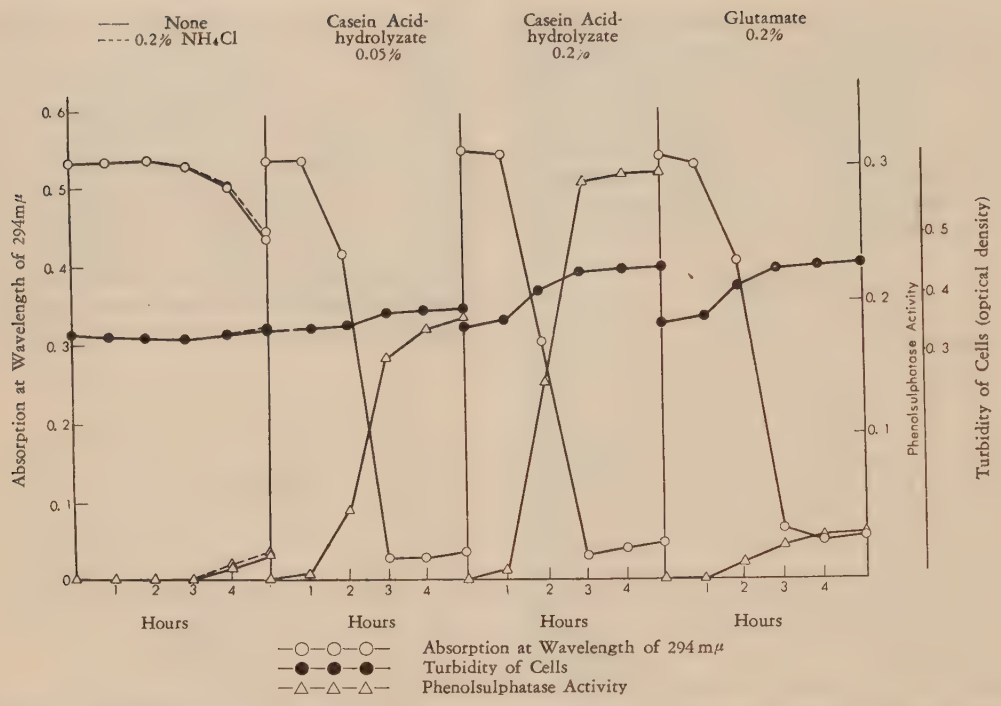

FIG. 2. The Effect of Nitrogen Sources on the Utilization of Tyramine and the Phenolsulphatase Synthesis by *Aerobacter aerogenes* 9621.

not metabolize tyramine immediately. Attempts were made to test the effect of several nitrogen compounds on the utilization of tyramine and the induction of PSase synthesis in them by shaking these cells with 5×10^{-3}M tyramine. This concentration of tyramine was shown to be optimal for PSase synthesis[2]. The results of a typical experiment are illustrated in Fig. 2. Decrease of tyramine, activity of PSase and growth of cells were plotted against time of incubation. With tyramine alone or tyramine plus ammonium chloride, both the utilization of tyramine and the PSase biosynthesis were very weak. In these cases, a very slight growth of cells was observed. These results indicated that ammonium chloride was unable to replace casein acidhydrolyzate. It seems very difficult for cell suspensions of *A. aerogenes*, to metabolize tyramine when it was supplied alone or even in the presence of inorganic ammonium salts, although considerable PSase activity had been obtained in tyramine medium for a long incubation period as previously reported.[2] When casein acidhydrolyzate, as the nitrogen source, was supplied with tyramine, PSase activity developed in response to the utilization of tyramine. The development of PSase activity was concerned with the concentration of casein acidhydrolyzate. When casein acidhydrolyzate was added to a 0.2 per cent concentration, a higher PSase activity developed, and tyramine was metabolized faster than in the case of 0.05 per cent concentration. But when it was increased to levels exceeding 0.2 per cent, both the utilization of tyramine and PSase synthesis were depressed.

The absorption spectra of the incubation mixture in alkaline state, which had been incubated with 0.2 per cent casein acidhydrolyzate and 5×10^{-3}M tyramine and were pipetted in time intervals of zero to 5 hours, are shown in Fig. 3. The typical absorption curve of tyra-

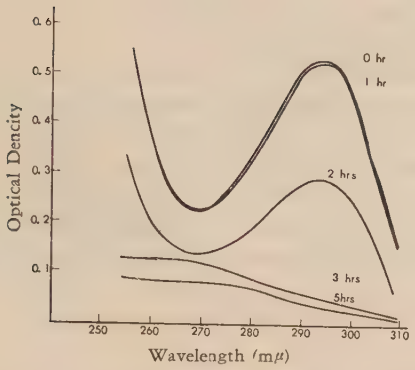

FIG. 3. The Absorption Spectra of Mixture Solution Containing Tyramine and Casein Acid-hydrolyzate in Various Incubation Times.

mine (or phenol) disappeared after 3 hours, and a broad absorption curve with low optical density appeared and seemed to increase its density. From this figure and also from the results of the paper chromatographic test of the incubation mixture, it is obvious that no phenolic compound was derived from tyramine, though some substances are accumulated in the incubation mixture. The latter broad absorption curve was not due to some compounds contained in casein acidhydrolyzate before incubation, since the absorption of 0.2 per cent casein acid-hydrolyzate was very weak as shown in Fig. 1. When 0.2 per cent glutamate was supplied, instead of casein acidhydrolyzate, with tyramine, a different figure was obtained as shown in the right column of Fig. 2. Although tyramine was utilized rapidly as in the case of the addition of 0.05 per cent casein acidhydrolyzate, PSase activity developed very slightly. Therefore it seemed that a mixture of amino acids would be necessary for both the rapid utilization of tyramine and the large PSase synthesis in addition to tyramine. Then the question arose, whether the organism could utilize amino acids as nitrogen sources as well as carbon and energy sources.

Effect of Carbon Sources on the Utilization of Tyramine and the PSase Biosynthesis.

Experiments were next carried out in order to find out the effect of carbon sources on the utilization of tyramine and PSase synthesis when carbon compounds were supplied with 5×10^{-3}M tyramine and 0.2 per cent casein acid-hydrolyzate. Table 1 shows the PSase activity of cells, the decrease of the absorption at the wavelength of 294 mμ and the growth of cells after 3 hours' incubation in various incubation mixtures.

The compounds tested as carbon sources might be divided into two groups though the effect gradually changed from one extreme (xylose) to another (rhamonse). One group (xylose etc.) was ineffective, slightly or rather moderately effective to the rapid utilization of tyramine and higher production of PSase caused by tyramine and casein acidhydrolyzate, while another group (glucose etc.) was remarkably inhibitory against both of these reactions, though the organism grew well in all of the cases. Xylose, glutamate, α-ketoglutarate, citrate, mannose, lactose, glycerol and inositol might be included in the former group, and fructose, gluconate, glucose, L-arabinose, galactose, mannitol and rhamnose might be considered as the latter group. D-Arabinose was unable to affect any reaction since this compound could not be utilized by the organism. These observed effects could not easily be correlated with any obvious chemical features of the compounds tested. However, this phenomena could be reasonably explained by "Diauxie"[5]. Magasanik[6] has indicated that the amount of L-histidine required to support the growth of a histidine less mutant of *A. aerogenes* depends on the nature of the major carbon source of the medium and that the carbon sources can be divided into two groups. And also, the glucose group has been shown to act inhibitor against the dissimilation of histidine. The compounds used in their experiments acted in a similar fashion to the utilization of tyramine and the PSase synthesis by the authors' experiments, with the exception of fructose and rhamnose. Our earlier studies with

5) J. Monod, Researches sur la croissance des cultures bacteriennes, Paris (1942).

6) B. Magasanik, *J. Biol. Chem.*, **213**, 557 (1955).

culture medium showed that glucose inhibited the formation of PSase by this strain. A similar finding had been made by Whitehead et al.[7]. They showed that no PSase could be detected in any of the tested strains of *Salmonella* and some strains of mycobacteria being considered to be PSase positive strains, when 0.5 per cent glucose was contained in culture medium. The reason for these inhibitions might be explained in various incubation periods with and without casein acidhydrolyzate.

As seen in Fig. 4, a remarkable delay of the inducing time for the utilization of tyramine and PSase synthesis was observed in the incubation mixture supplied with glucose both in the presence and absence of casein acidhydrolyzate in comparison with that supplied with xylose. Further, in the absence of casein acidhydrolyzate,

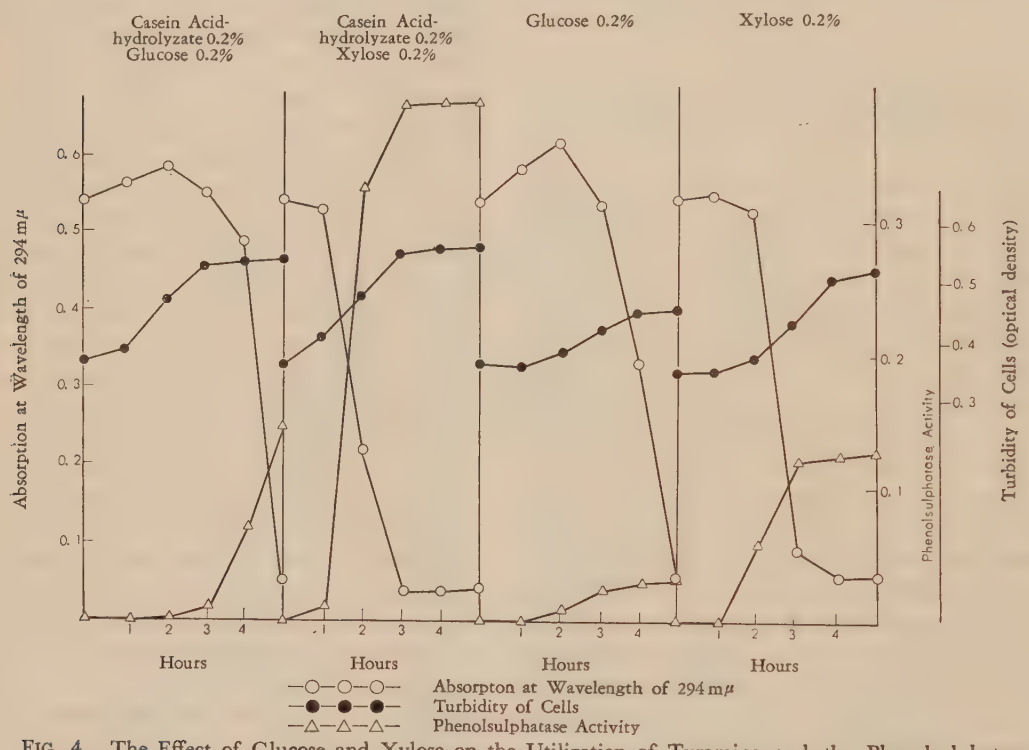

FIG. 4. The Effect of Glucose and Xylose on the Utilization of Tyramine and the Phenolsulphatase Synthesis in the Presence and Absence of Casein Acidhydrolyzate by *Aerobacter aerogenes* 9621.

by the same mechanism.

From these results it was found that any substance inhibiting the dissimilation of tyramine, also inhibited PSase synthesis.

In order to clarify their relations more clearly, the effects of glucose and xylose, as the representatives of two groups on the utilization of tyramine and PSase synthesis, were investigated

7) J. E. M. Whitehead, A. R. Morrison and L. Young, *Biochem. J.*, **51**, 585 (1952).

growth of bacteria when supplied with glucose, was shown to be much poorer than in the case of xylose. These facts indicated that in the presence of casein acidhydrolyzate, tyramine might be utilized after the disappearance of glucose, this being reasonably explained by the "Diauxie" phenomenon and that in the absence of casein acidhydrolyzate glucose is less effective as a primer for the utilization of tyramine than xylose.

TABLE I. THE EFFECT OF VARIOUS CARBON SOURCES ON THE UTILIZATION OF TYRAMINE AND THE PSASE SYNTHESIS IN THE PRESENCE OF CASEIN ACIDHYDROLYZATE BY *Aerobacter aerogenes*.

Compounds	Phenolsulphatase Activity	Decrease of Absorption at wavelength of 294 mμ	Turbidity of Cells (optical density)
Xylose	0.38	0.50	0.20
Glutamate	0.30	0.50	0.18
α-Ketoglutarate	0.30	0.50	0.14
D-Arabinose	0.31	0.50	0.10
None	0.30	0.50	0.10
Citrate	0.22	0.33	0.13
Mannose	0.12	0.12	0.14
Lactate	0.09	0.19	0.12
Glycerol	0.09	0.15	0.12
Inositol	0.09	0.20	0.15
Fructose	0.045	−0.09	0.13
Gluconate	0.04	0.04	0.11
Glucose	0.025	−0.25	0.17
L-Arabinose	0.02	−0.04	0.16
Galactose	0.008	−0.027	0.14
Mannitol	0.007	−0.043	0.08
Rhamnose	0.005	−0.025	0.14

In the case of glucose, the absorption optical density at the wavelength of 294 mμ increased after incubation for one or two hours. However, this increase of absorption was found to appear at wavelength ranging from 250 mμ to 310 mμ, approximately at in the same rate. Therefore, this increase might be due to the appearance of some compound derived mainly from glucose under these conditions. On addition of each member of the glucose group, a similar attitude was observed (Table I). In the case of xylose group members, no such increase of absorption occurred. This may be explained that tyramine might be dissimilated as fast as xylose or other compounds.

It was also demonstrated that PSase activity becomes slightly higher, when xylose is added in the incubation mixture which consisted of tyramine and casein acidhydrolyzate. The reason for this has not yet been clarified. When xylose was added with tyramine, but without casein acidhydrolyzate, the dissimilation of tyramine was delayed to a certain extent and the activity of PSase was about 40 per cent in comparison with the case of both tyramine and casein-hydrolyzate.

From the comparison of experimental data obtained from a series of combination of elementary compounds, such as tyramine only, tyramine plus xylose, tyramine plus glucose, tyramine plus glutamate, tyramine plus casein acidhydrolyzate, the following conclusion was made. The carbon of tyramine can be utilized easily provided a suitable primary carbon source is supplied. Mixture of amino acids such as casein acidhydrolyzate is the best for both the utilization of tyramine and PSase synthesis. The formation of PSase by this organism starts with the utilization of tyramine. *A. aerogenes* seems to have a weak or either no activity in utilizing tyramine without a primary carbon or "primer".

PSase Synthesis by Tyramine Adapted Cells.

In order to ascertain whether external substances are necessary for PSase synthesis by tyramine adapted cells, first of all various concentrations of casein acidhydrolyzate were tested for their effect in supporting the utilization of tyramine and PSase synthesis. Tyramine adapted cells were obtained by the following

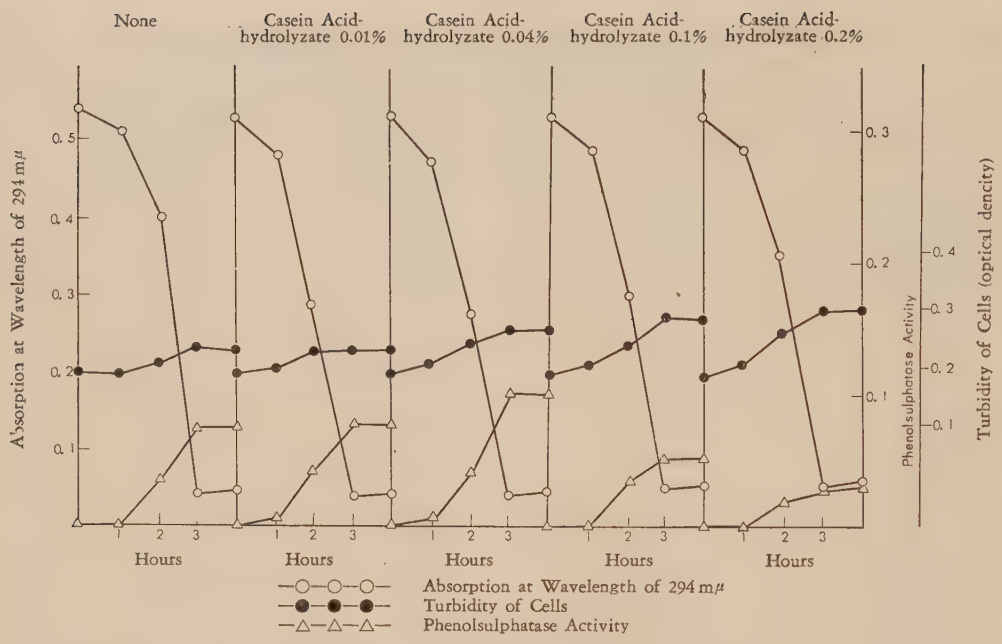

FIG. 5. The Effect of Casein Acidhyrolyzate on the Utilization of Tyramine and the PSase Synthesis by Tyramine Adapted Cells of *Aerobacter aerogenes* 9621.

TABLE II. THE EFFECT OF SOME CARBON SOURCES ON THE UTILIZATION OF TYRAMINE AND THE PSASE SYNTHESIS BY TYRAMINE ADAPTED CELLS OF *Aerobacter aerogenes*.

Compounds	Concentration (per cent)	Phenolsulphatase Activity	Decrease of Absorption at Wavelength of 294 mμ
None		0.070	0.49
Casein Acidhydrolyzate	0.01	0.075	0.49
	0.04	0.100	0.48
	0.1	0.060	0.48
	0.2	0.025	0.48
Glutamate	0.01	0.035	0.48
	0.04	0.025	0.47
	0.1	0.020	0.47
	0.2	0.015	0.47
Xylose	0.01	0.060	0.49
	0.04	0.055	0.48
	0.1	0.040	0.48
	0.2	0.035	0.48
Glucose	0.01	0.060	0.49
	0.04	0.055	0.49
	0.1	0.030	0.31
	0.2	0.025	0.23

method: Cells harvested as above mentioned from the culture medium, were washed and suspended in a solution containing 5×10^{-3}M tyramine and 0.2 per cent glutamate with phosphate buffer. The incubation mixture was aerated for one hour and a quater. The cells were

devoid of recognizable PSase activity. Then, the cells were harvested and washed twice with distilled water. The adapted cells were subsequently aerated with 5×10^{-3}M tyramine and varying concentrations of casein acidhydrolyzate.

Fig. 5 indicates that although a small amount of PSase was synthesised without casein acidhydrolyzate, the activity of PSase was increased by the addition of 0.01 or 0.04 per cent casein acidhydrolyzate. However, in the case of a larger amount of casein acidhydrolyzate (0.1 or 0.2 per cent), a considerable inhibition against the PSase synthesis was observed. When xylose, glucose or glutamate was added in varying concentrations instead of casein acidhydrolyzate, inhibition against PSase synthesis occurred in every case, even in a very dilute concentration. From these experiments, it was apparent that a small amount of external amino acids promoted the synthesis of PSase as well as the utilization of tyramine by the tyramine adapted cells. Other carbon sources tested were not considered necessary for the utilization of tyramine and were rather inhibitory against the PSase synthesis.

DISCUSSION

Previous studies indicated that tyramine induced the PSase synthesis of *A. aerogenes* and its specificity as an inducer was very high. More recently, working with *Salm. schottmuelleri*, the author also observed a remarkable effect of tyramine on the PSase synthesis of this microorganism. Both species of bacteria were capable of dissimilating tyramine. Furthermore, in the culture of many species of *Aspergillus* and *Penicillium* similar effects of tyramine were found although a considerable amount of PSase developed even when tyramine was not supplied. It was also confirmed that these molds could dissimilate tyramine. On the other hand, in many species of *Rhizopus* having an ability to form PSase but incapable of dissimilating tyramine, no effect of tyramine has yet been observed. Such an effect was not found in *Str. alcalophilus*, the principal microorganism which caused the coloration of lignin in putryfying urine[8, 9]. A study based on this mold will be published in the future. Accordingly, it seems probable that tyramine would affect PSase synthesis only by action of the microorganisms having capacity of dissimilating tyramine. These findings have lead the author to investigate whether or not oxidative dissilation of tyramine is always necessary for the development of PSase by many microorganism species.

In the present study, the relationship between the utilization of tyramine and the PSase synthesis was investigated by cell suspensions of *A. aerogenes* when various substances were supplied in addition to tyramine. From experiments with this organism, it was found that tyramine could hardly be utilized and a very small amount of PSase was formed unless amino acids or other carbon sources were supplied. PSase was synthesized in response to the utilization of tyramine which was caused by the addition of appropriate substances with tyramine. When various carbon sources were supplied with tyramine and casein acidhydrolyzate, several compounds such as glucose inhibited not only the utilization of tyramine but also PSase synthesis, while other compounds such as xylose were ineffective or only a little effective to both reactions. Therefore, this possibility was considered that the oxidative dissimilation of tyramine might be necessary for PSase synthesis. However the fact whether any metabolism of tyramine is concerned with PSase synthesis has not been ascertained in this study.

In any case, PSase could develop only when tyramine was metabolized in the case of this strain, although great differences in PSase activity developed by the effect of various substances which were added with tyramine were recognized. Among many of these compounds, casein acidhydrolyzate proved to be most effective for PSase synthesis in both cases of tyramine-adapted and nonadapted cells. These facts indicated that amino acids, as well as serving as

8) T. Harada, *J. Agr. Chem. Soc. Japan*, **26**, 95 (1952).
9) T. Harada, *J. Agr. Chem. Soc. Japan*, **29**, 516 (1955).

nitrogen sources, might play a role as sparkers or primers of tyramine dissimilation for the enzyme synthesis.

Acknowledgement: The author wishes to express his sincere thanks to Prof. Z. Nikuni for his kind advice and suggestions in the course of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 230~233 1959]

Studies on Phenolic Lactones

Part II. A New Synthetic Method of Isohibalactone

By Kyôhei YAMASHITA and Masanao MATSUI

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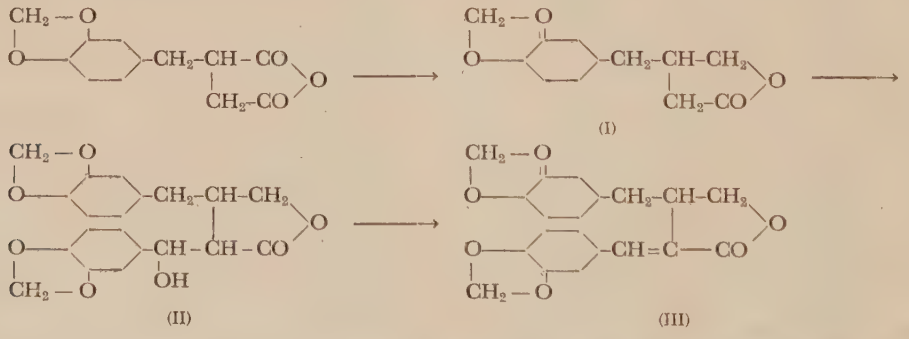
Received September 16, 1958

Isohibalactone, the geometric isomer of hibalactone, was synthesized by the following route. Piperonylsuccinic acid anhydride was converted into thioethyl methyl ester ans was reduced to piperonylbutyrolactone by Raney nickel catalyst. Piperonylbutyrolactone was also prepared from piperonylsuccinic anhydride by the reduction with amalgamated aluminum. Condensation of piperonal with the lactone in the presence of potassium amide afforded α-(3,4-methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-butyrolactone, m.p. 151~2°C. Dehydration of the hydroxylactone with *p*-toluenesulfonic acid gave isohibalactone, m.p. 156~6.5°C.

In the previous paper[1] the authors reported the synthesis of α-piperonylidene-β-piperonylbutyrolactone from the corresponding succinic acid. But the product was not identical with hibalactone in its infrared spectra and the authors designated it as isohibalactone. As was described there, during the reduction of thioethylester with Raney nickel catalyst most of the double bond was also hydrogenated and the yield of unsaturated lactone was rather poor. Therefore the authors deviced an another route to α-piperonylidene-β-piperonyl-butyrolactone.

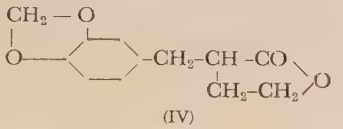


(I) (II) (III)

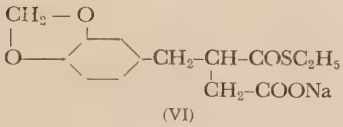
For the synthesis of β-piperonylbutyrolactone

1) K. Yamashita and M. Matsui, This Bulletin, **22**, 227 (1958).

(I) two methods were employed; one was the Raney nickel reduction of thioethyl methyl ester and the other was the amalgamated aluminum reduction of acid anhydride. In both cases α-piperonylbutyrolactone (IV) may be produced accompanied with β-piperonyl-butyrolactone (I). As the amalgamated aluminum reduction of ethyl succinic anhydride afford β-methylbutyrolactone, β-piperonylsuccinic anhydride would assumed to be reduced to β-piperonylbutyrolactone. In the other preparations as the reaction of sodium mercaptide with acid anhydride would afford either (V) or (VI), the resulting lactones would be α-substituted lactone or β-substituted lactone respectively.



(IV)



(VI)

When piperonal was condensed with γ-butyrolactone (IV) α-disubstituted lactone (VII) would be the only product and it could not be dehydrated to unsaturated lactone. In practise the condensation product could be dehydrated to α,β-unsaturated lactone, so the starting butyrolactone was confirmed to have the structure of β-substituted lactone.

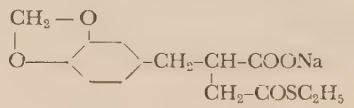
Piperonal was condensed with the lactone in the presence of potassium amide in liquid ammonia. The product was confirmed to be α-(3,4-methylenedioxyphedyl - hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-γ-butyrolactone by its elementary analysis and infra red spectra. Dehydration of hydroxylactone with *p*-toluenesulfonic acid gave α-piperonylidene-β-piperonyl-γ-butyrolactone, and the obtained lactone was identical with isohibalactone previously obtained differed from hibalactone.

The authors express their thanks to Dr. Mitsuo Masumura, Tokushima University, for kind gift of authentic hibalactone.

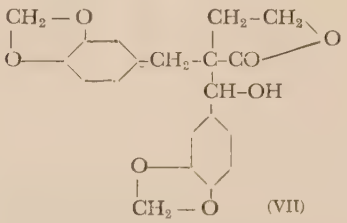
EXPERIMENTAL

β-(3,4-Methylenedioxybenzyl)-butyrolactone

a) To a suspension of 3.5 g of sodium sand in 150 ml of benzene was added 25 g of ethylmercaptan and agitated several hours until all the sodium was converted into ethylmercaptide. Piperonylsuccinic anhydride (m.p. 105~6°C, prepared from 27.8 g of piperonylsuccinic acid) in 150 ml of dry benzene was added to above suspension and stirred 3 hours at room temperature and left standing overnight. The reaction mixture was extracted thrice with each 100 ml of 10% aqueous sodium



(V)



(VII)

carbonate solution. The combined extract was acidified with dil. sulfuric acid in cold and extracted four times with benzene. The combined extract was washed with water and the solvent was distilled off. Monothioethylester was obtained as viscous oil.

Crude monothioethylester was dissolved in 200 ml of ether and esterified with etherial solution of diazomethane prepared from 20 g of nitrosomethylurea. Evaporation of the solvent left 31.5 g of methyl-thioethyl ester as a syrup. The crude product was directly used in the following reduction.

Raney Ni catalyst was prepared from 70 g of alloy by the method of Adkins. In a 1-l three-necked flask were placed 300 ml of 70% ethylalcohol and 35 g of Raney nickel catalyst. A solution of 31.5 g of methyl-thioethyl ester of piperonylsuccinic acid in 150 ml of aqueous alcohol was added dropwise and stirring was continued for 4 hours at 50°C and left standing overnight. The filtered solution was concentrated to syrup in vacuo. The residual oil was refluxed with 200 ml

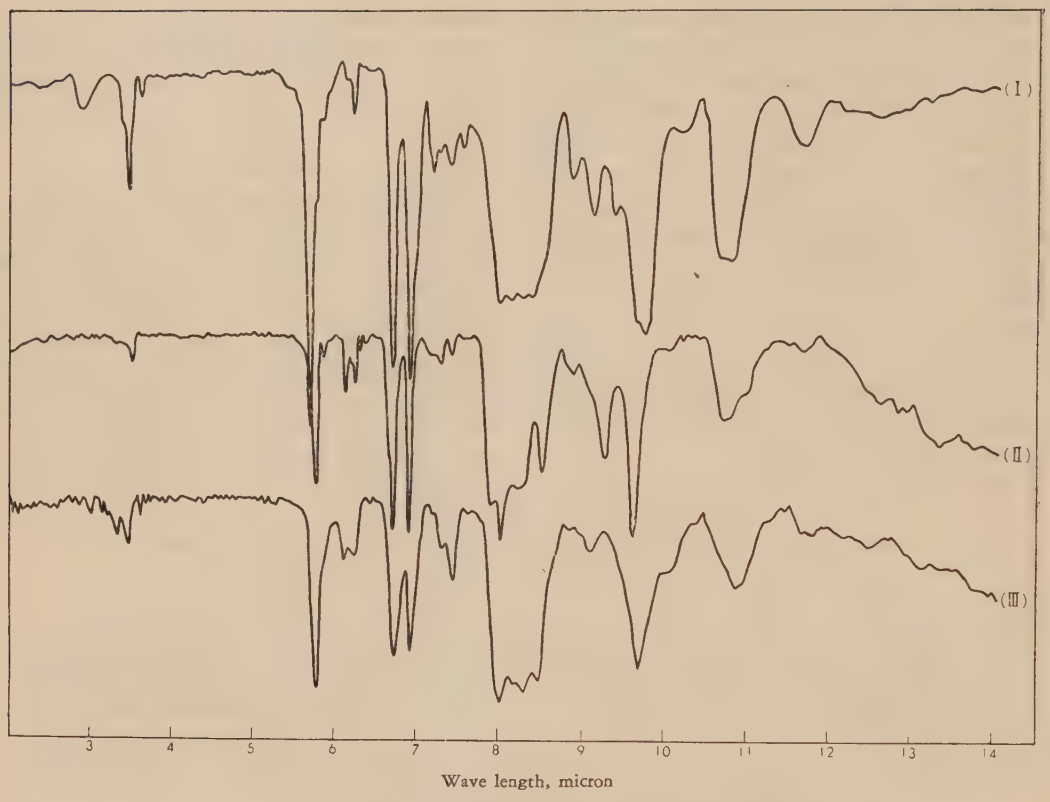


FIG. 1. Infrared Absorption Spectra (Chloroform solution)

(I) α-(3, 4-methylenedioxyphenyl-hydroxymethyl)-β-(3, 4-methylenedioxybenzyl)-butyrolactone
(II) isohibalactone
(III) hibalactone (authentic)

of 10% methanolic potassium hydroxide solution for an hour. After the addition of water (200 ml) removal of methanol and neutral substances were removed by ether extraction. The mother liquor was acidified with dil. hydrochloric acid and warmed on a water bath for 20 minutes. The mixture was poured into excess of aqueous sodium bicarbonate solution then with water and dried over anhydrous sodium sulfate. Evaporation of the solvent left 6.4 g of oily lactone.

b) Piperonyl succinic acid was converted by the treatment with acetic anhydride to the corresponding anhydride, m.p. 104–5.5°C. The acid anhydride (5 g) was dissolved in a mixture of benzene (100 ml) and ether (100 ml) and mixed with amalgamated aluminum (12 g), and water gradually added to the mixture during 12 hours. After filtration the residue was extracted with acetone; evaporation of the combined extract and filtrate yielded an oil, which was refluxed with 5% methanolic potash (50 ml) for an hour. The methyl-alcohol was removed, and the filtered solution acidified with hydrochloric acid and heated at 100° for half an hour. Excess of sodium bicarbonate was added and after digestion on the water bath for 30 minutes the mixture was extracted with chloroform. The combined extract was washed with water; evaporation of the solvent left 2.4 g of oily lactone.

α-(3, 4-Methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-butyrolactone.

Metalic potassium (2.7 g) was dissolved in 100 ml of liquid ammonia in the presence of a trace of ferric nitrate. After all the potassium was converted into potassium amide, the mixture of 5.2 g of piperonal and 7.5 g of β-piperonyl-γ-butyrolactone on 20 ml of ether was added dropwise, and left standing overnight in acetone-dryice mixture. Dried ether (100 ml) was added and the ammonia was evaporated at room temper-

ature. Methanol (100 ml) was added and ether was removed by distillation, then 5 ml of water was added and refluxed for an hour. Water (50 ml) was added and the methanol was distilled off. Neutral substances were removed by ether extraction and the aqeous solution was acidified with hydrochloric acid and warmed on water bath for 30 minutes. The reaction mixture was poured into excess aqueous sodium bicarbonate solution and extracted with chloroform. The extract was washed with water and dried over anhydrous sodium sulfate. Evaporation of the solvent left 8.6 g of oily product. Upon standing for several days on refrigerator white needles was gradually separated, m.p. 147~8°, yielded 3.5 g. Recrystallization from methanol gave colorless prisms, m.p. 152°. *Anal.* Found: C, 64.89; H, 4.93. Calcd. for $C_{20}H_{18}O_7$ requires: C, 64.9; H, 4.8%. From the elementary analysis and infra red spectrum this was assumed to be α-(3,4-methylenedioxyphenyl-hydroxymethyl)-β-(3,4-methylenedioxybenzyl)-butyrolactone.

α-Piperonylidene-β-piperonyl-γ-butyrolactone (Isohibalactone).

The hydroxylactone (1 g) was dissolved in 100 ml of xylene and a small amount of *p*-toluenesulfonic acid was added. After refluxing for an hour the mixture was washed with aqueous sodium bicarbonate solution and water. Evaporation of the solvent left 0.9 g of crystalline mass, m.p. 151~2°C. Recrystallization from methanol gave colorless prisms, m.p. 156~156.5°C. *Anal.* Found: C, 68.22; H, 4.81. Calcd. for $C_{20}H_{16}O_6$: C, 68.1; H, 4.5%. The mixed melting point with isohibalactone was also 156°C.

The hydroxylactone (1 g) was dissolved in 10 ml of acetic anhydride and refluxed for an hour. Distillation of acetic anhydride left 0.9 g of crystalline mass. Recrystallization from methanol afforded white pellet, m.p. 115~6°. *Anal.* Found: C,6 4.02; H, 4.92. Calcd. for $C_{22}H_{20}O_8$ requires: C, 64.1; H, 4.8%. The obtained lactone was obviously acetylated hydroxylactone from the analytical value and infra red spectrum.

[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 233~239 1959]

Wood Extractives

Part VIII. On the Heartwood Constituents of *Cryptomeria japonica* D. Don. (1)

By Tamio Kondo, Hiroyuki Imamura and Motoshige Suda

Government Forest Experiment Station, Meguro, Tokyo

Received September 25, 1958

Petroleum ether extract of the heartwood of *Cryptomeria japonica* D. Don contains isodextropimaric acid, sugiol, xanthoperol, and a viscous resin. After saponification of the resin fraction, β-sitosterol, ferruginol, xanthoperol and an unknown compound are found to be present, showing the occurrence of these substances in states of chemical combination with other constituents. It has now been proposed that isodextropimaric acid is optically active.

The Japanese genus *Cryptomeria*, principally *C. japonica* D. Don, which causes endemic in East Asia, is the source of the most popular timber commonly known as "sugi" in Japan. *C. japonica* is a coniferous tree large in size belonging to the Family Pinaceae and is planted throughout this country, except in Hokkaido. The wood is used for constructional purposes and as a source for various technological utilities. In view of application its effects on the al-

dehyde-contents of Japanese wine obtained from wine wood extractives[1,2] and on the chemical constituents of its essential oil[3,4] have been examined. Early systematic work on the resinous exudate from the tree was carried out by S. Keimatsu et al.[5] and G. Hukui et al.[6]. They isolated two compounds, namely, cryptopimaric acid, $C_{20}H_{30}O_2$, from its acid fraction and sugiol, $C_{20}H_{28}O_2$, from its phenol fraction, respectively. Progress to a certain extent was accomplished towards the elucidation of the structures of both compounds by these Japanese workers.

The tree has two types of heartwood by its color, e.g., the one is called red heartwood and the other black heartwood; the former is preferred as a woodworking material. However, so far no definite explanation has been presented on the development of both types of heartwood coloration.

On woodworking application, especially on the production of plywood and veneer, the surface board consisting of this timber takes stains which are more frequently bluish dark when set in contact with an iron vessel.

According to recent field investigation[7], the heartwood of *C. japonica* is considered to be a kind of wood most resistant to termites.

As it is pointed out in some reviews concerning wood extractives[8,9,10], these characteristics in wood property will be reasonably explained by the presence of some chemical compounds in the woody tissue. Hitherto, in spite of early research on the extractives from *C. japonica*[4,11], so far no chemically homogeneous constituent has been isolated from the timber. The object of the present work is to isolate some extractable substances from the tree and identify individual compounds.

Specimens obtained from the so-called red heartwood of the tree were used in the form of saw dust. Treatment with boiling methanol yielded considerable amounts of a reddish brown resinout extract, as much as ca. 6% of the heartwood being removed in this way. Further treatment of the methanol extract with cold petroleum ether gave a yellow solution, which was divided into three fractions, e.g., acidic, phenolic and neutral potions by shaking with aqueous 2% sodium carbonate followed by 2% sodium hydroxide solutions in the usual manner. The sodium soluble-fraction gave a diterpenoid resin acid (I), $C_{20}H_{30}O_2$, m.p. 166–168°, $[\alpha]_D^{25}$ −22.0°, identical with cryptopimaric acid as designated by S. Keimatsu et al.

The alkali soluble-fraction afforded two crystalline products, e.g., a phenolic diterpenoid ketone (III), sugiol, $C_{20}H_{28}O_2$, m.p. 298–299° (decomp.), $[\alpha]_D^{29}$ +30.6°, and a phenolic diterpenoid diketone (IV), xanthoperol, $C_{20}H_{26}O_3$, m.p. 255–277° (decomp.), $[\alpha]_D^{22}$ +142.6°. Identification of both tricyclic diterpenoid phenols were conducted through preparations of some characteristic derivatives respectively. Since the neutral fraction exhibited a distinct maximum at ca. 280 mμ in the ultraviolet absorption spectrum in methanol, it was supposed that this fraction might consist of some related phenolic diterpenes. After saponification with alcoholic potassium hydroxide, this fraction was treated with steam distillation until no further oily distillate was observed, and subsequently chromatographed on alumina using petroleum ether, ether and then methanol as eluants. The petroleum ether eluate afforded a light yellow viscous liquid, which was acetylated with pyridine and acetic anhydride, rechromatographed on alumina eluted initially with petroleum ether and subsequently with ether. The main fraction of the petroleum ether eluate was identified as ferruginol (II) by tedious procedure, as described in the following experimental section.

Elution with methanol yielded an unknown

1) S. Yamada, *J. Agr. Chem. Soc. Japan*, **1**, 818 (1925); **4**, 155 (1928).
2) T. Azuma, *Bull. Inst. Phys. & Chem. Research, Tokyo*, **7**, 763 (1928); **8**, 831 (1929).
3) H. Kimura, *J. Pharm. Soc. Japan*, **33**, 2 (1913).
4) S. Keimatsu, *J. Pharm. Soc. Japan*, **26**, 315 (1907).
5) S. Keimatsu et al., *ibid.*, **57**, 69 (1937)
6) G. Hukui et al., *ibid.*, **59**, 124 (1939).
7) T. Shibamoto, Annual Meeting of Japan Wood Res. Soc. (1958).
8) T. Kondo, *Wood Ind. Tokyo*, **12**, 555 (1957).
9) D. Narayanamurti, *Holz Roh- u. Werkstoff*, **15**, 370 (1957).
10) W. Sandermann et al., *ibid.*, **15**, 281 (1957).
11) I. Miura et al., *Bull. Tokyo Univ. Forests*, No. 15 (1931).

compound, m.p. 182°, $[\alpha]_D^{25}+3.9°$, for which the formula $C_{20}H_{34}O$ was tentatively proposed.

A considerable amount of β-sitosterol was found to be present in the ether eluate portion of the saponified neutral-fraction.

The yellow band on the alumina chromatogram that developed during ether elution, was eluted with methanol and gave a yellow crystalline compound (IV), xanthoperol, which has been discovered in the wood extractive from common juniper (*Juniperus communis* L.) and its chemical structure elucidated by J.B. Bredenberg[12, 13].

This author, J.B. Bredenberg, observed that xanthoperol was first obtained from the hydrolysed neutral-fraction, a fact which seemed to indicate that the product was an artefact. As seen in Fig. 1, it was a matter of interest to find that a small quantity of xanthoperol exists in a free state on the heartwood of *C. japonica*.

The isolation scheme of wood extractives mentioned above is elucidated in Fig. 1, showing the yields of individual components, expressed in terms of weight of air dry wood.

The resin acid obtained from the sodium soluble-fraction has been supposed by D.H.R. Barton[14] and by H. Erdtman[15] to be possibly identical with isodextropimaric acid, and is now under rigid investigation. The melting points and other properties of the acid, dihydroderivative, and methyl ester corresponded to those of isodextropimaric acid and its related derivatives. It is of interest to find the optical activities of

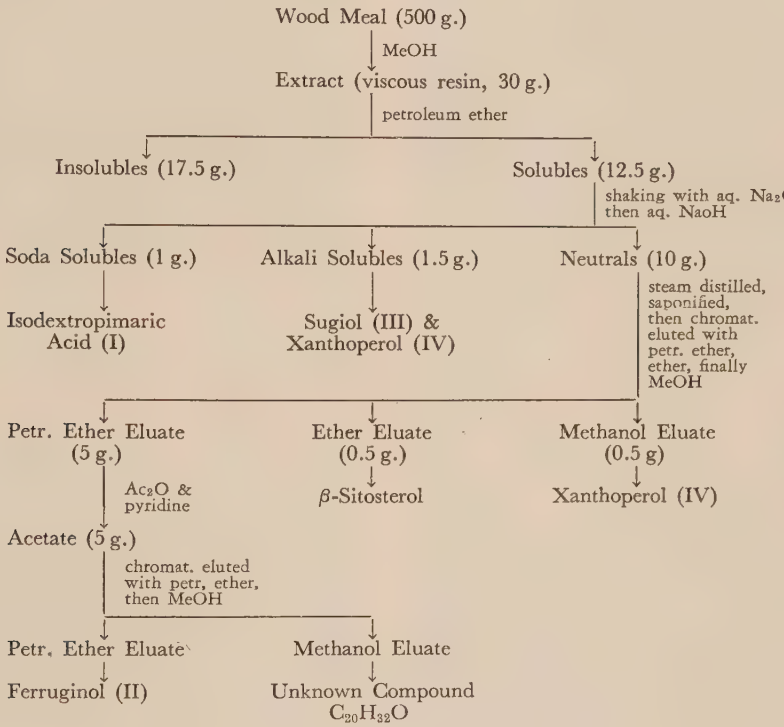


FIG. 1. Isolation Scheme of Extractives.

12) J. B. Bredenberg and J. Gripenberg, *Acta Chem. Scand.*, **10**, 1511 (1956).
13) J. B. Bredenberg, *ibid.*, **11**, 927 (1957).
14) J. W. Cook, (Ed), Progress in Orgenic Chemistry, I, 22 (1952).
15) E. H. Rodd, (Ed.), Chemistry of Carbon Compounds, IIB, 712 (1953).

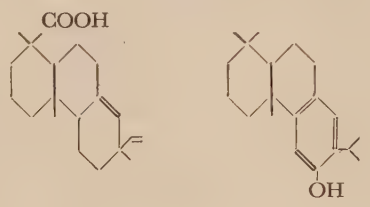
TABLE 1.

	m.p.	$[\alpha]_D$
Isodextropimaric acid from *C. Japonica*	166–168	−20.00
Dihydro acid	173–175	+26.08
Methyl ester	63– 64	−25.65
Butanolamine salt	186–188	−32.00
Cyclohexylamine salt	197–199	−25.80

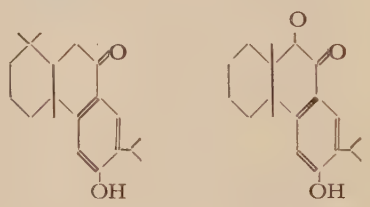
the acid and its related compounds given in Table I. Of these activities, those of the methyl ester and dihydroderivative have already been pointed out by C. Ukita et al.[16].

It is found here that isodextropimaric acid is optically active showing $[\alpha]_D$ −20°, practically identical with data for cryptopimaric acid presented by S. Keimatsu et al. This result suggests a fact showing that isodextropimaric acid, when obtained by G.C. Harris' procedure[17], possibly becomes optically inactive. It seems probable that isodextropimaric acid, originally having a small definite laevorotatory action, undergoes racemization to the inactive form in the course of G.C. Harris' isolation process. This finding was confirmed through comparison of its IR spectrum with a specimen obtained from pine oleoresin using G.C. Harris' procedure, and with the characteristic absorption bands for isodextropimaric acid reported by H.H. Bruun[18].

The conclusion that isodextropimaric acid is present in an optical active form was further substantiated by the fact that miropinic acid, which was identified as isodextropimaric acid by O. Jeger et al.[19], has been reported to have laevorotatory activity, $[\alpha]_D$ −3.6°.



(I) (II)



(III) (IV)

EXPERIMENTAL

Unless otherwise indicated elsewhere, methanolic solutions were used for ultraviolet absorption measurements.

Isolation and Separation

(1) Isolation of Isodextropimaric Acid, Sugiol and Xanthoperol.

"Sugi" heartwood was extracted three times with boiling methanol for five hr.; evaporation of the solvent from the methanolic extract combined left a viscous residue. Repeated extraction of this residue with cold petroleum ether afforded a yellow transparent solution, which was shaken with 2% aqueous sodium carbonate, and then 2% aqueous sodium hydroxide solutions. The acidic terpene was isolated from the sodium-fraction, by acidification and ethyl acetate-extraction, as a yellowish brown crystal. The acid (I) was conveniently purified via its easily crystallizable cyclohexylamine salt.

The phenolic terpene was similarily isolated from the alkali fraction, and chromatographed on alumina. Elution with ether afforded a colorless crystal (III), and developed a yellow distinct band on the upper layer of the column. This band was eluted with methanol, giving yellow needles (IV) on removal of the solvent. In contrast with Bredenberg's finding on the occurrence of xanthoperol as an artefact, a small quantity of the product was found to be present in a free state on the heartwood of the tree.

(2) Isolation of β-Sitosterol and Xanthoperol.

The neutral fraction, which was left by evaporation of the solvent from the petroleum ether solution after shaking with alkali, was boiled with 10% alcoholic potassium hydroxide for five hr. The majority of the used alcohol was then removed by evaporation in a water bath, and the residue was extracted with ethyl acetate. The acetate solution was shaken with an excess of water until no alkaline reaction was defined, dried over sodium sulfate and evaporated. The residue was dissolved in petroleum ether and the solution was run on alumina and washed with large amounts of petroleum ether, ether, and then methanol. Petroleum ether eluted

16) C. Ukita et al., *J. Pharm. Soc., Japan*, **72**, 1324 (1952).
17) G. C. Harris et al., *J. Am. Chem. Soc.*, **70**, 2079 (1948).
18) H. H. Bruun, *Paper and Timber*, **39**, No. 12 (1956).
19) A. Brossi and O. Jeger, *Helv. Chim. Acta*, **33**, 722 (1950).

a mixture of ferruginol (II) and an unknown diterpene compound, as mentioned below. The ether eluant gave β-sitosterol as a colorless compound. Elution with ether developed a yellow band which eluted using methanol, and on evaporation gave yellow needles (IV). By means of this operation, red heavy crystals, m.p. 217–223° (decomp.) were occasionally obtained in the mixture with yellow needles (VI). The red compound was considered to be an Al-complex of (IV), because it was decomposed with alcoholic sulfuric acid, sprayed up, added with water, and regenerated to yellow needles (IV).

(3) Isolation of Ferruginol and an Unknown Compound.

The aforementioned petroleum ether eluate was purified three times through vacuum distillation (bath-temp. 200–220°/0.2–0.3 mm.Hg), treated with pyridine-acetic anhydride over night at room temperature, poured into an excess of water, and extracted with ethyl acetate. The residue of the acetate solution was dissolved in petroleum ether and rechromatographed on alumina. Petroleum ether eluted crude ferruginol acetate and subsequently, methanol eluted an unknown compound.

Isodextropimaric Acid (I).

Yellowish brown crystals regenerated from the sodium-fraction were crystallized several times from aqueous methanol and fine needles were obtained. Further purification was made via its cyclohexylamine salt by the same procedure as adopted by G.C. Harris et al. Fine needles, m.p. 166–168°, $[\alpha]_D^{25}$ −20.0°. Found: C, 79.42; H, 10.00. Calcd. for $C_{20}H_{30}O_2$: C, 79.14; H, 9.97%. Ultraviolet absorption: $\lambda_{max.}$ 212 mμ (log ε, 3.89). Infrared absorption: $\lambda_{max.}^{Nujol}$ 1818(w), 1681(s), 1634(w), 1524(w), 1412(m), 1348(w), 1319(w), 1280(s), 1250*(sh.), 1217(w), 1188(m), 1152*(sh.), 1145*(w), 1022(w), 997(m), 990(m), 938(m), 907*(m), 800(m), 733*(w), 716*(w), (* denotes the characteristic bands for isodextropimaric acid reported by H. H. Bruun[18]).

Cyclohexylamine salt of the resin acid crystallized from ethanol yielding colorless needles, m.p. 197–199° (decomp.). $[\alpha]_D^{12}$ −25.80° (c, 0.85 in MeOH). Found: C, 77.75; H, 10.91. Calcd. for $C_{26}H_{43}O_2N$: C, 77.75; H, 10.79%.

Butanolamine salt of the acid crystallized from acetone and then ethyl acetate yielded colorless needles, m.p. 186–188° (decomp.), $[\alpha]_D^{12}$ −32.0° (c, 1.00 in MeOH).

Dihydroisodextropimaric acid was obtained by catalytic hydrogenation in ethanol solution in the presence of palladium charcoal catalyst. The dihydro compound crystallized from aqueous ethanol yielded plates, m.p. 173–175°, $[\alpha]_D^{30}$ +26.08° (c, 1.00 in MeOH). Found: C, 78.80; H, 10.77. Calcd. for $C_{20}H_{32}O_2$: C, 78.89; H, 10.59%.

Methyl isodextropimarate prepared upon treatment with diazomethane, recrystallized from ethanol in prisms, m.p. 63–64°, $[\alpha]_D^{16}$ −25.65° (c, 0.97 in MeOH). Found: C, 79.88; H, 9.95. Calcd. for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19%.

Mixed melting point determinations of the acid and its derivatives with a corresponding authentic specimen obtained in the same way as described by G. C. Harris et al.[16] were carried out and, no depression was observed in all cases.

Sugiol (9-Ketoferruginol) (III).

Colorless crystals obtained from the ether eluate were purified by repeated crystallization from ethanol and then by sublimation two times, under reduced pressure to afford fine needles, m.p. 298–299° (decomp.) $[\alpha]_D^{29}$ +30.6° (c, 0.65 in pyridine). Found: C, 79.80; H, 9.16. Calcd. for $C_{20}H_{28}O_2$: C, 79.95; H, 9.39%. Ultraviolet absorption: $\lambda_{max.}$ 232, 283, 304(sh.) mμ (log ε, 4.08, 3.99, 3.93(sh.)), Infrared absorption: $\lambda_{max.}^{Nujol}$ 3145(m), 3096(m), 1634(s), 1587(s), 1572(s), 1506(m), 1460(m), 1385(m), 1353(m), 1318(s), 1274(s), 1247(m), 1182(m), 1088(w), 869(w), 771(w).

On treatment with pyridine-acetic anhydride and by recrystallization from aqueous ethanol, it gave the monoacetyl derivative as tough prisms, m.p. 160–162°. Found: C, 77.28; H, 8.76. Calcd. for $C_{22}H_{30}O_3$: C, 77.15; H, 8.83%.

Methylation of the product in acetone solution with dimethyl sulfate in the presence of anhydrous potassium carbonate gave the monomethyl derivative as colorless fine needles, m.p. 132–134°. Found: C, 80.22; H, 9.65. Calcd. for $C_{21}H_{30}O_2$: C, 80.21; H, 9.62%.

The oxime, prepared by boiling the product with hydroxylamine hydrochloride in the presence of pyridine, crystallized readily from benzene giving plates, m.p. 177–179° (decomp.). Found: C, 76.95; H, 8.85. Calcd. for $C_{20}H_{29}O_2N$: C, 76.20; H, 9.27%.

The melting points of the phenol and its derivatives were in agreement with values stated in the literature[6].

Xanthoperol (IV).

Elution of the yellow band with methanol gave yellow crystals, m.p. 225–277° (decomp.) after recrystallization from ethanol and sublimation under reduced pressure. Owing to the lack of the product, the authors could only prepare one derivative, monoacetyl xanthoperol, by means of the pyridine-acetic anhydride method. The

acetate formed yellowish needles (from aqueous ethanol), and melted at 151–152°, either alone or mixed with the specimen obtained from the neutral fraction described below.

On saponification with alcoholic potassium hydroxide solution and then by alumina chromatography, the neutral fraction afforded a considerable amount of xanthoperol. Purification was carried out in a way similar to that described previously. Yellow prisms, m.p. 255–277° (decomp.), $[\alpha]_D^{22}$ +142.6°. Found: C, 76.49; H, 8.26. Calcd. for $C_{20}H_{26}O_3$: C, 76.40; H, 8.34%. Ultraviolet absorption: $\lambda_{max.}$ 254, 357–358 mμ (log ε, 3.70, 4.03). Infrared absorption: $\lambda_{max.}^{KBr}$ 3413(s), 2967(s), 2907(sh.), 2865(sh.), 1709(s), 1656(s), 1595(s), 1572(s), 1508(m), 1475(s), 1439(w), 1403(w), 1383(w), 1342(s), 1297(s), 1271(s), 1208(m), 1186(m), 1166(m), 1151(m), 1109(m), 1087(w), 1049(m), 917(m), 885(m), 869(m).

The monoacetyl derivative was prepared by treatment with pyridine-acetic anhydride and crystallized from aqueous ethanol and yielded yellowish needles m.p. 151–152°, identical (m.p. and mixed m.p.) with the corresponding compound described above. Found: C, 74.13; H, 7.61. Calcd. for $C_{22}H_{28}O_4$: C, 74.13; H, 7.92%.

The monomethyl derivative, prepared with dimethyl sulfate and anhydrous potassium carbonate, crystallized from aqueous ethanol and yielded faint yellow needles, m.p. 186–187°. Found: C, 76.92; H, 8.62. Calcd. for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59%.

Treatment of this methyl ether in absolute ethanol containing pyridine with hydroxylamine hydrochloride and crystallization from aqueous methanol furnished the mono-oxime, faint yellow needles, m.p. 164–165°. Found: C, 73.48; H, 8.53. Calcd. for $C_{21}H_{29}O_3N$: C, 73.43; H, 8.51%.

Quinoxaline derivative of xanthoperol was prepared by heating in glacial acetic acid solution with an equal weight of *o*-phenylenediamine, fixation of the product on alumina column and then by elution with methanol, yielded very faint yellowish needles (from aqueous methanol), m.p. 262–264° (decomp.). Found: C, 80.76; H, 7.88. Calcd. for $C_{26}H_{30}ON_2$: C, 80.79; H, 7.82%.

The melting points of the mother compound and its derivatives were in accordance with those reported by J.B. Bredenberg[12].

β-Sitosterol.

The ether eluate from the original chromatogram furnished a crystalline compound in a good yield. The product was repeatedly crystallized from ethanol as scale-like crystals, m.p. 135–136.5°, giving a positive Liebermann-Burchard reaction. Found: C, 83.67; H, 12.16. Calcd. for $C_{29}H_{50}O$: C, 83.99; H, 12.15%.

Monoacetate had m.p. 121–122°. Found: C, 81.55; H, 11.44. Calcd. for $C_{31}H_{52}O_2$: C, 81.52; H, 11.48%.

The monobenzoate formed scale-like crystals, m.p. 143–144° (from a mixture of ethanol and benzene). Found: C, 83.42; H, 10.40. Calcd. for $C_{36}H_{54}O_2$: C, 83.34; H, 10.49%.

The *p*-nitrobenzoate formed fine needles, m.p. 184–185° (from a mixture of ethanol and chloroform). Found: C, 76.73; H, 9.31. Calcd. for $C_{36}H_{54}O_4N$: C, 76.69; H, 9.48%.

Occurrence of Ferruginol.

The syrupy acetyl derivative of the original petroleum ether eluate was rechromatographed on alumina and eluted with petroleum ether, and then with methanol. The former eluant gave crude ferruginol, and the latter, an unknown diterpenoid described below. In spite of their efforts, the present authors failed to separate monoacethyl ferruginol in the crystalline state from crude acetate fraction. However, oxidation of this fraction with chromic acid in an acetic acid solution in the water bath and isolation of the neutral portion in the usual manner, gave monoacetyl sugiol in a good yield, which separated as prisms from aqueous ethanol, m.p. and mixed m.p. 160–162°. Accordingly, this fraction was rechromatographed in the usual way after being refluxed with alcoholic potassium hydroxide solution. The petroleum ether eluate was purified by distillation and fractionation in vaccum, and then by rechromatography. In the present chromatography, the main fraction could not be eluted with petroleum ether, but was first obtained as a colorless liquid with ether as the eluant. This liquid was identified as ferruginol through the preparation of crystalline monobenzoyl derivative. The material was treated with pyridine-benzoyl chloride overnight and the products, isolated in the usual way, were chromatographed twice on alumina, and then distilled (bath-temp. 210–230°/0.3 mm.Hg). The distillate solidified and crystallized from a mixture of benzene and ethanol yielding plates, m.p. 152.5–153.5. Found: C, 83.10; H, 8.98. Calcd. for $C_{27}H_{34}O_2$: C, 83.03; H, 8.78%. For the monobenzoyl ferruginol, C. W. Brandt et al. recorded m.p. 154°[20].

An Unknown Compound.

The further methanol eluate mentioned above gave a crystalline product which after crystallization from aqueous ethanol, had m.p. 181–182°, $[\alpha]_D^{15}$ +14.52° (c,

20) C. W. Brandt and L. G. Neubauer, *J. Chem. Soc.*, **1939**, 1031.

0.76 in MeOH). The product was obtained as colorless needles being soluble in warm alcohols, acetone, and chloroform, and gave a negative Liebermann-Burchard reaction. We proposed a tentative formula, $C_{20}H_{34}O$ for this product. Found: C, 83.03; H, 11.60. Calcd. for $C_{20}H_{34}O$: C, 82.69; H, 11.80%.

Further studies of this product are now in progress.

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[Bull. Agr. Chem. Soc. Japan, Vol. 23, No. 3, p. 239~243 1959]

Studies on 5′-Phosphodiesterases in Microorganisms

Part I. Formation of Nucleoside-5′-monophosphates from Yeast Ribonucleic Acid by *Penicillium citrinum**

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During investigations conducted on the mode of degradation of ribonucleic acid by Microorganisms, it was found that one strain of *Penicillium* produces nucleoside-5′-monophosphates from ribonucleic acid. Identification of these nucleoside-5′-monophosphates was carried out by means of chromatography on anion exchange column, ultraviolet spectrophotometry, paper electrophoresis, and characteristic color reactions using orcinol, carbazole, and rosaniline. It is suggested that this strain contains 5′-phosphodiesterase (or 5′-phosphodiesterase system) which cleaves all of the 5′-phosphodiester linkages in ribonucleic acid thus giving rise to nucleoside-5′-monophosphates. It is a point of interest in this study to indicate the presence of 5′-phosphodiesterase for the first time in a common microorganism, as 5′-phosphodiesterase has been recognized only in somewhat particular materials such as snake venom.

Recently, it has become evident that the ribonucleic acids are polynucleotides in which individual nucleoside residues are joined, one to the other by phosphodiester linkages between the 3′- and 5′-positions[1]. When ribonucleic acids are depolymerized, cleavage must be considered to occur at either the 3′-phosphodiester linkages ($C_3'-O-P(O_2H)\dashv O-C_5'$) or at the 5′-phosphodiester linkages ($C_3'-O\dashv P(O_2H)-O-C_5'$). Since Kunitz[2] first obtained pancreatic ribonuclease in crystalline form, many papers on enzymes degrading ribonucleic acids have been published. Most of these enzymes split 3′-phosphodiester linkages in ribonucleic acids giving rise to nucleoside-2′, 3′-cyclic phosphates or nucleoside-3′-monophosphates. The mode of the action of these enzymes appear to resemble that of alkali. Only phosphodiesterases of snake

* Presented at the Commemorative Meeting of Nippon Shoyu Kyokai, Tokyo, July 9, 1957, and at the Meeting of the Kanto Division of the Agricultural Chemical Society of Japan, Tokyo, June 28, 1958.

1) D. M. Brown and A. R. Todd, "The Nucleic Acids", (ed. E. Chargaff and J. N. Davidson), Academic Press. Inc. New York, 1955, Vol. 1, p. 409.

2) M. Kunitz, *J. Gen. Physiol.*, **24**, 15 (1940).

venom[3,4], intestinal mucosa[5,6], and rye grass[7,8] have been demonstrated to split 5′-phosphodiester linkages in ribonucleic acid giving rise to nucleoside-5′-monophosphates.

In the course of investigating ribonucleolytic enzymes of various microorganisms, the authors have found that the mycelium of a strain of *Penicillium citrinum* contained 5′-phosphodiesterase or the 5′-phosphodiesterase system, which specifically cleaves all of the 5′-phosphodiester linkages in yeast ribonucleic acid* giving rise to nucleoside-5′-monophosphates.

The present communication deals with the characterization of the degradation products formed by the action of *Penicillium* mycelium upon yeast ribonucleic acid. The detailed properties of *Penicillium* 5′-phosphodiesterase will be discussed in the next paper.

EXPERIMENTAL

1. Separation of the Nucleotides formed by the Action of *Penicillium* Mycelium upon Yeast Ribonucleic Acid.

Fifty ml of an aqueous culture medium in each 150-ml Fernbach flask containing 5% glucose, 0.5% polypeptone, 0.05% monobasic potassium phosphate, 0.05% dibasic potassium phosphate, 0.04% magnesium sulfate, and 0.04% calcium chloride, pH 5.6, was sterilized and inoculated with a strain of *Penicillium*, *Penicillium citrinum* Thom 1131**. After surface culture at 30°C for five days the mycelial deck was separated from the culture broth, and washed with sterilized water. The washed mycelial deck was then secondarily incubated with 50 ml of 0.5% yeast ribonucleic acid solution containing 0.01 N sodium fluoride at 30°C. Experiments without using either ribonucleic acid or mycelial deck, were carried out simultaneously. After 22.5 hr. the deck was removed. The amount of material absorbing ultraviolet excreted during incubation from the mycelium was less than 6.5% of ribonucleic acid employed as the substrate. Uranyl reagent-soluble phosphates[9] in the resulting reaction mixture were determined according to the Fiske-Subbarow method[10]. As described in Table I, approximately 66.5% of ribonucleic acid was depolymerized. Approximately 56.6% of the resultant uranyl reagent-soluble phosphates were further decomposed to inorganic phosphate.

TABLE I. FORMATION OF URANYL REAGENT-SOLUBLE PHOSPHATES FROM RIBONUCLEIC ACID† BY *Penicillium* MYCELIUM

No.	Composition of reaction mixture	Uranyl reagent-soluble P (μg/ml) formed during incubation		
		Total P	Inorg. P	Org. P
1	RNA+NaF+Mycelium	282.0	154.4	127.6
2	RNA+NaF	6.4	0	6.4
3	NaF+Mycelium	10.0	4.0	6.0
	(1)−{(2)+(3)}	265.6	150.4	115.2

Experimental conditions are described in the text.
† contained 400 μg of RNA-P per ml of solution.

Most of the uranyl reagent-soluble organic phosphates may be regarded as mononucleotides. To identify these mononucleotides, a nucleotide fraction was prepared from the reaction mixture as follows: 23 ml of the reaction mixture was adjusted to pH 8.5 with strong sodium hydroxide solution. A quantity of 2.5 ml of 20% barium acetate solution was added thereto. The supernatant was adjusted to pH 5.0 with a small quantity of acetic acid. One ml of mercuric acetate solution (20% in 2% acetic acid) was then added. The precipitate was centrifuged, washed and suspended in water. Into the suspension hydrogen sulfide gas was introduced so as to separate free nucleotides. The mixture was filtered and the precipitate was washed with hot water. The solution resulting from the washing was combined with the supernatant. A portion of the combined solution, equivalent to 465 μg phosphorus, was adjusted to pH 8.5, and charged into anion exchange resin Dowex-1-Cl-X-4 (200–400 mesh) column, with a diameter of 1.0 cm and 23 cm in height, and was eluted with 0.003 N (Eluate No. 1−No. 216) and 0.010 N hydrogen chloride (Eluate No. 217−No. 302). Each 80 drops of the eluate was collected into a test tube and optical density of each eluate was read at 260 mμ, and five ultraviolet-absorbing fractions-A, B, C, D, and E were obtained.

2. Characterization of Each Nucleotide

By means of paper chromatography using 2 solvent

3) J. M. Gulland and E. M. Jackson, *J. Chem. Soc.*, **1938**, 1492.
4) W. E. Cohn and E. Volkin, *J. Biol. Chem.*, **203**, 319 (1953).
5) W. E. Cohn and E. Volkin, *Nature*, **167**, 483 (1951).
6) W. E. Cohn, D. G. Doherty, and E. Volkin, "Phosphorus Metabolism", (ed. W. D. McElroy and B. Glass), Baltimore, 1952, Vol. 2, p. 339.
7) L. Shuster and N. O. Kaplan, *J. Biol. Chem.*, **201**, 535 (1953).
8) L. Shuster, *J. Biol. Chem.*, **229**, 289 (1957).
* Yeast ribonucleic acid was supplied by Kirin Research Institute, and used without further purification.
** This strain was supplied by the Institute of Applied Microbiology, University of Tokyo.
9) A. Kuninaka, *J. Gen. Appl. Microbiol.*, **3**, 55 (1957).
10) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

systems[11], each fraction was observed to include only one ultraviolet-absorbing phosphorus compound corresponding to nucleotide. Characterization of each compound was carried out as follows. (The results are summarized in Table II)

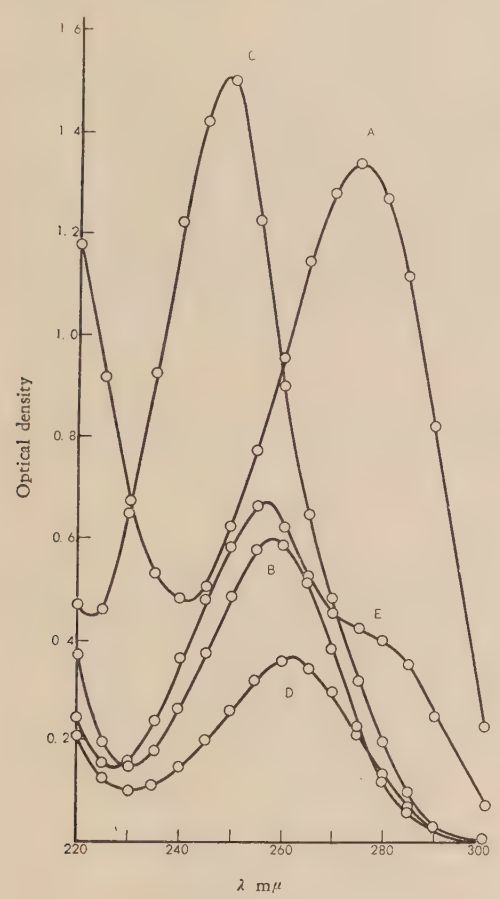

FIG. 1. Ultraviolet Absorption Spectra of the Eluates No. 22 (A)†, No. 50 (B), No. 122 (C), No. 228 (D), and No. 271 (E).

† Before reading the optical densities, the solution was diluted with an equal volume of water.

Ultraviolet Spectrophotometry The ultraviolet absorption spectra of fractions A, B, C, D, and E shown in Fig. 1, were identical with spectra of cytidine-, adenosine-, inosine-, uridine-, and guanosine-phosphates, respectively.

11) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **29**, 797 and 801 (1955).

TABLE II. CHARACTERIZATION OF THE NUCLEOTIDES FORMED FROM RIBONUCLEIC ACID BY *Penicillium citrinum*

	Fraction	Eluate No.	λ max† (mμ)	% Color development (Pentose-orcinol reaction[12]) 7 min.	15 min.	25 min.	35 min.	45 min.	Carbazole reaction[13]	Distance migrated from origin to the anode side by electrophoresis†† (cm)	Periodate oxidation[14]
Nucleotide fraction obtained	A	21– 24	275							4.9	+
	B	43– 54	258	78.7	94.2	100.0	96.0	93.9	blue	4.9	+
	C	121–122	249	82.2	98.9	100.0	96.8	92.6	blue	12.5	+
	D	221–237	262							14.7	+
	E	264–281	257	81.6	97.2	100.0	96.3	92.8	blue	7.7	+
Standard substance	Cytidine-3′-monophosphate†††		278							4.9	–
	Adenosine-3′-monophosphate†††		257						purple	5.0	–
	Adenosine-5′-monophosphate		257	79.0	94.4	100.0	96.4	92.8	blue	4.5	+
	Inosine-5′-monophosphate		250	80.4	95.7	100.0	95.4	92.1	blue	12.5	+
	Uridine-3′-monophoshate†††		262							15.0	–
	Guanosine-3′-monophosphate†††		257	42.5	80.2	98.3	100.0	99.1	purple	8.8	–
	Mixture of 3′-nucleotides†††		258	39.6	72.8	94.7	99.0	100.0	purple	14.8, 8.7, 4.7	–

† Ultraviolet absorption spectra of standard substances were measured in 0.1N HCl.

†† Paper electrophoresis was performed in 10% acetic acid with 400 V and 0.23–0.30 m amp/cm at room temperature for 4.5 hr. The starting line was set at 5 cm from the end of the cathode side, and 26 cm from the end of the anode side.

††† Preparations of nucleoside-3′-monophosphates employed in the present study probably contained the corresponding 2′-isomers respectively.

The Ratio of the Base Component to the Phosphate Component In each nucleotide fraction, the ratio of the base component determined ultraviolet-spectrophotometrically to the phosphate component was found to be approximately 1.0. It therefore appears that the compound of each fraction was nucleoside-monophosphate being neither di- nor tri-phosphate.

Orcinol Reaction An aliquot of each purine nucleotide fraction (B, C, E) equivalent to $0.1-0.2$ μmol. of each nucleotide was heated with the orcinol reagent according to the method of Albaum and Umbreit[12]. The rates of color development with fractions B, C, and E were recognized to be essentially similar to the rate of development with inosine-5′-monophosphate or adenosine-5′-monophosphate, and could be distinguished from the rate of guanosine-3′-monophosphate or a mixture of various nucleoside-2′- or 3′-monophosphates. As for the rate of color development in orcinol reaction, the nucleotides in fractions B, C, and E were not considered to be either nucleoside-2′- or 3′-phosphates tub 5′-phosphates.

Carbazole Reaction Carbazole reaction was carried out with an aliquot of each concentrated purine nucleotide fraction (B, C, E). The technique employed here was essentially the same as that described by Dische and Landsberg[13]. The greenish blue color developed by each fraction was quite identical with the color developed with adenosine-5′-monophosphate or inosine-5′-monophosphate, and was clearly distinguishable from the purple color developed with purine nucleoside-3′-monophosphates. As for the carbazole reaction, also the nucleotides in fractions B, C, and E were not considered to be either nucleoside-2′- or 3′-phosphates but 5′-phosphates.

Paper Electrophoresis and Periodate Oxidation An aliquot of each concentrated nucleotide fraction was subjected to paper electrophoresis in 10% acetic acid[9,11]. Each fraction was observed to include one ultraviolet-absorbing component corresponding to each nucleotide. After the detection of ultraviolet-absorbing spots*, periodate oxidation combined with Schiff's reaction was carried out directly on the paper by application of the method of Buchanan, Dekker and Long[14]. As shown in Table II, the nucleotides obtained from ribonucleic acid by *Penicillium* mycelium were oxidized by periodate indicating that these compounds are not nucleoside-2′- or 3′-phosphates but 5′-phosphates.

All of the above data show that the ultraviolet-absorbing phosphorus compounds which are contained in fractions A, B, C, D, and E are cytidine-, adenosine-, inosine-, uridine-, and guanosine-5′-monophosphates, respectively. Nucleoside diphosphates and oligonucleotides were not detected in this experiment. It may be concluded that the mycelium of *Penicillium citrinum* contain 5′-phosphodiesterase which cleaves all of the 5′-phosphodiester linkages in yeast ribonucleic acid giving rise to cytidine-, adenosine-, guanosine,- and uridine-5′-monophosphates. Inosine-5′-monophosphate may be formed from adenosine-5′-mono-phosphate by the action of deaminase. The properties of the 5′-phosphodiesterase of *Penicillium citrinum* are under investigation by means of the cell free preparation. As the cell free preparation does not need the presence of inorganic phosphate to split ribonucleic acid, it is apparent that this enzyme is not a polynucleotide phosphorylase but a hydrolase.

DISCUSSION

The enzymes catalyzing degradation of ribonucleic acid may be classified on the basis of their mechanism of degradation and the kind of linkages on which the enzymes act. This classification is shown as follows:

- Ribonucleo-phosphodiesterase
  - 3′-Phosphodiesterase
    - Pyrimidine nucleoside-3′-phosphodiesterase—— Pancreas (RNase I)
    - Purine nucleoside-3′-phosphodiesterase—— Takadiastase (RNase T)[15]
    - Unspecific 3′-phosphodesterase—— Spleen, Tobacco leaf[16]
  - 5′-phosphodiesterase
    - Unspecific 5′-phosphodiesterase—— Snake venom[3,4], Intestinal mucosa[5,6], Rye grass[7,8], *Penicillium citrinum*
- Polynucleotide phosphorylase

It may be suggested that the enzyme found by the authors in *Penicillium citrinum* belongs to 5′-phosphodiesterase group. Among the enzymes belonging to this group, the so-called "unspecific"

12) H. G. Albaum and W. W. Umbreit, *J. Biol. Chem.*, **167**, 369 (1947).
13) Z. Dische and E. Landsberg, *Biochim. et Biophys. Acta*, **24**, 193 (1957).
* The authors wish to express their thanks to Dr. Iwase, the Scientific Research Institute, for kindly supplying ultraviolet filter-2537 Å for detection of ultraviolet-absorbing spots.
14) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, **1950**, 3162.
15) K. Sato and F. Egami, *J. Biochem. Japan*, **44**, 753 (1957).
16) K. K. Reddi *Biochim. et Biophys. Acta*, **28**, 386 (1958).

phosphodiesterases of snake venom and intestinal mucosa hydrolyze not only all of the 5'-phosphodiester linkages in ribonucleic acid, but also various phosphodiester linkages in other phosphodiesters such as deoxyribopolynucleotides and diphenyl phosphate. The "ribonuclease" of germinating rye grass also seems to split all of the 5'-phosphodiester linkages in ribonucleic acid. It is still an uncertain problem whether the 5'-phosphodiesterase activity of *Penicillium citrinum* is due to specific enzymes or to the presence of an unspecific diesterase. At the time when the specificity and other enzymatic characteristics of this *Penicillium* 5'-phosphodiesterase will be revealed in detail, this enzyme may be used effectively for study on the structure of ribonucleic acid.

Snake venom, intestinal mucosa, and rye grass, which contain 5'-phosphodiesterases acting on ribonucleic acid, are considered to be somewhat particular materials in biochemical field. Furthermore, snake venom and intestinal mucosa also contain strong phosphomonoesterase. Therefore, nucleoside-5'-phosphates, which play a more important biochemical role than nucleoside-2'- or 3'-phosphates, have been considered not to be derived from ribonucleic acid but from coenzymes[17]. Generally speaking, the nucleotides derived from ribonucleic acid are nucleoside-3'-(or 2'-) phosphates, which are inferior to nucleoside-5'-phosphates in respect of their biochemical action. However, our observations clearly demonstrate that 5'-phosphodiesterase activity does exist in a common microorganism like *Penicillium citrinum*. It may be suggested that in some microorganisms, nucleoside-5'-monophosphates are derived not only from coenzymes but also from ribonucleic acid. In these organisms nucleoside-5'-phosphates seem to have relation to both synthesis and the degradation of ribonucleic acid, and there might be some direct relations among the metabolism of ribonucleic acid, nucleoside-5'-monophosphates and coenzymes. Recently, it has been clarified that, likewise in animals, nucleoside-5'-phosphates are widely distributed in microorganisms[18,19,20,21,22]. Consequently, it seems probable that these nucleoside-5'-phosphates have relation to the metabolism of both coenzymes and ribonucleic acid. It is a matter of special interest that various nucleoside-5'-phosphates have been found in *Penicillium chrysogenum*[22].

17) E. L. Oginsky and W. W. Umbreit, "An Introduction to Bacterial Physiology", W.H. Freeman, San Francisco, 1954, p. 290.
18) J. Baddiley and A. P. Mathias, *Chemistry and Industry*, **1954**, 277.
19) H. Schmitz, *Biochem. Z.*, **325**, 555 (1954).
20) J. W. Newton, J. B. Wilson and P. W. Wilson, *J. Bact.*, **69**, 677 (1955).
21) A. L. Koch, *J. Biol. Chem.*, **203**, 227 (1953).
22) A. Ballio, C. Casinovi and G. Serlupi-Crescenzi, *Biochim. et Biophys. Acta*, **20**, 414 (1956).

Microbiological Hydroxylation of Steroids by *Absidia regnieri*

Sir:

In microbial hydroxylations of steroids, the introduction of the 14-hydroxy group is a common reaction caused by molds, especially by the species of Mucor[1].

We have also found that a strain of *Absidia regnieri* carries out the 14α-hydroxylation of steroids. When progesterone was used as the steroid substrate in the fermentation of *Absidia regnieri*, 14α-hydroxyprogesterone was obtained as the monohydroxyderivative of the steroid substrate.

But besides this monohydroxylation, this fungus was also capable of introducing, at the same time, two or three hydroxyl groups into progesterone.

The introduction of three hydroxyl groups at the same time into the steroid nucleus rarely occurs in microbial hydroxylation.

Hereupon, in the present communication we wish to report on the preparation of 14α-hydroxyprogesterone, 6β, 14α-, 7α, 14α-dihydroxyprogesterone and 7α,14α,15β-trihydroxyprogesterone by *Absidia regnieri*.

When Reichstein's compound S (17α, 21-dihydroxy-4-pregnene-3,20-dione) was used instead of progesterone in fermentation of this fungus, it was converted predominantly to epi-hydrocortisone (11α, 17α, 21-trihydroxy-4-pregnene-3,20-dione), making only minor amounts of the 14α-hydroxyderivative.

This result is particularly interesting in respect of stereochemical specificity of substrates on the microbial hydroxylation of steroid.

In our experiment reported here, *Absidia regnieri* was grown on a reciprocating shaker for 48 hours at 26° in 10 l of a medium of the following composition: 5% glucose, 2% pepton and 0.5% corn steep liquor.

Then, 5 grams of progesterone was added in a methanol solution and fermentation was allowed to proceed for additional 48 to 72 hours.

The culture was filtered and the oxydized steroids were recovered from the filtrate by ethyl acetate extraction followed by washing with 2% sodium bicarbonate solution and water. The ethyl acetate solution was evaporated to dryness in vacuo and redissolved in chloroform and the insoluble matter was removed by filtration. The product obtained by recrystallization of this insoluble matter from acetone was 6β, 14α-dihydroxyprogesterone (I), m.p. 242–246°; $[\alpha]_D$+132° (pyridine); $\lambda_{max.}^{alc.}$ 236 mμ (ε=13,800). The monoacetate of (I), m.p. 175.5–177.5°, $[\alpha]_D$ +101.8° (pyridine).

Reduction of (I) by mild treatment with zinc and acetic acid afforded a known-14α-hydroxyprogesterone.

The chloroform solution of the residue was chromatographed on Florisil and developed by chloroform-acetone solvent mixtures.

The major product eluted in the first fraction was 14α-hydroxyprogesterone (II), m.p. 192–194°; $[\alpha]_D$+197° (chloroform); $\lambda_{max.}^{alc.}$ 240 mμ (ε=17,050).

From the second fraction was yielded 7α, 14α-dihydroxyprogesterone (III), m.p. 212–216°; $[\alpha]_D$ +141° (chloroform); $\lambda_{max.}^{alc.}$ 242 mμ (ε=16,200). The monoacetate of (III), m.p. 191.5–192.5°; $[\alpha]_D$+161.0° (chloroform). The crystallization of the last group of fractions afforded trihydroxyprogesterone (IV), m.p. 267–269° (dec.); $[\alpha]_D$+75.1° (pyridine); $\lambda_{max.}^{alc.}$ 239.5 mμ (ε=16.020). The infrared spectrum of (IV) was idential to that of 7α, 14α, 15β-trihydroxyprogesterone[2] obtained

1) P. D. Meister, S. H. Eppstein et al., *J. Am. Chem. Soc.*, **80**, 3383 (1958).

2) K. Tsuda, E. Oki et al., Monthly Meeting of Pharm. Soc. Japan (Tokyo). Dec. 6, 1958.

by a strain of *Syncephalastram* sp.

Some other unidentified dihydroxyprogesterone was also obtained in a small amount.

When Reichstein's compound S was used as asteroid substrate in similar techniques, most of the oxygenated product obtained was epi-hydrocortisone (V), m.p. 206–209°; $[\alpha]_D$+120° (methanol), $\lambda_{max.}^{alc.}$ 242 mμ (ε=14,500) and a minor amount of 14α,17α,21-trihydroxy-4-pregnene-3,20-dione (VI) was isolated, m.p. 213–218°; $[\alpha]_D$+155° (methanol); $\lambda_{max.}^{alc.}$ 241 mμ (ε=11,000). The 21-mono-acetate of (VI) was, m.p. 222–227° $[\alpha]_D$+177° (chloroform). From these results, this fungus has considerable stereochemical specificity of steroid substrate in hydroxylation, that is, it appears that 14α-hydroxylation by this fungus is affected profoundly by the presence of 17α-hydroxyl or the 21-hydroxyl function.

The authors wish to express their thanks to Professor K. Tsuda and Professor T. Asai, of the Institute of Applied Microbiology, University of Tokyo, and Mr. M. Matsui, Director of this Laboratory for their helpful advice and constant guidance throughout this work.

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Microbiological Hydroxylation of Progesterone and 17α-Hydroxyprogesterone by *Sclerotinia libertiana*; 2β-Hydroxylation.

Sir:

Among the numerous hydroxylation of steroids by microorganisms, 2β-hydroxylation has only recently been discovered[1].

In the previous paper[2] we have reported on a microbial hydroxylation of Reichstein's compound S (17α-hydroxydesoxycorticosterone) to 2β,17α,21-trihydroxy-4-pregnene-3,20-dione utilizing *Sclerotinia libertiana*.

In the present communication we wish to report on the microbial hydroxylation of progesterone and 17α-hydroxyprogesterone by this fungus in the 2β-position.

When progesterone was used as the steroid substrate, the fermentation of this fungus yielded 2β,15β-dihydroxyprogesterone and several unidentified dihydroxyprogesterones.

In case 17α-hydroxyprogesterone was used, 2β,17α-dihydroxy-, 15β,17α-dihydroxy- and 11α,17α-dihydroxyprogesterone were produced.

Among these isolated compounds, 2β,15β-dihydroxy- and 2β,17α-dihydroxyprogesterone have been recognized to be hitherto- undescribed new compounds.

In the experiment reported here, *Sclerotinia libertiana* was grown aerobically on a reciprocating shaker at 26° for 48 hours in 10 l of a medium of potatodecoct containing 3% of glucose.

Five grams of the requisite steroid was then added in methanol solution and the fermentation was allowed to proceed for a further 72 hours. The oxygenated products were extracted with ethyl acetate from the whole broth and the extract was first washed with 2% sodium bicarbonate solution and then with water. The ethyl acetate solution was then taken to dryness in vacuo and the residue was dissolved in chloroform. This chloroform solution was chromatographed on Florisil or alumina. Chloroform-acetate solvent mixtures were used as the developer.

When progesterone was incubated with *Sclero-*

1) H. L. Herzog, *J. Am. Chem. Soc.*, **79**, 3921 (1957). G. Greenspan et al., *ibid.*, **79**, 3922 (1957).
2) M. Shirasaka et al., *Bull. Agr. Chem. Soc. Japan*, **22**, 273 (1958).

tinia libertiana, 2β, 15β-dihydroxyprogesterone (I) was isolated as the major product; m.p. 206–216°; $[\alpha]_D$ −66.7° (chloroform); $\lambda_{max.}^{MeOH}$ 243 mμ (ε=15,080); $\lambda_{max.}^{KBr}$ 1700 cm^{-1}, 1671 cm^{-1} and 1621 cm^{-1}.

Anal. Found. C, 72.82; H, 8.92. Calcd. for $C_{21}H_{30}O_4$: C, 72.80 H, 8.73%. The diacetate of (I), m.p. 127–128°; $[\alpha]_D$ −51.3° (chloroform). The monoacetate of (I), m.p. 137–138°; $[\alpha]_D$ −22.7° (chloroform).

The structure of (I) has been assigned on the basis of molecular rotation, the characteristic change of ultraviolet absorption in alkaline solution and the behavior by treatment with zinc and acetic acid affording the known 15β-hydroxyprogesterone. The other three unidentified dihydroxyprogesterones were also obtained in this experiment.

If 17α-hydroxyprogesterone was used as the steroid substrate with this fungus *Sclerotinia libertiana*, 2β, 17α-dihydroxyprogesterone (II) was isolated. m.p. 219–221°; $[\alpha]_D$ −125° (chloroform); $\lambda_{max.}^{MeOH}$ 243 mμ (ε=16,000). *Anal.* Found: C, 72.43; H, 8.28. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73%.

The monoacetate of (II), m.p. 187.5–188.5°. The structure of this new compound (II) has been decided on the basis described above.

Besides this, a small amount of 15β, 17α-dihydroxyprogesterone (III), m.p. 258–259° $[\alpha]_D$ +54.3° (chloroform); $\lambda_{max.}^{MeOH}$ 240.5 mμ (ε=15,000) and 11α, 17α-dihydroxyprogesterone (IV), m.p. 216–218°; $[\alpha]_D$ +73° (chloroform); $\lambda_{max.}^{MeOH}$ 241.5mμ (ε=17,000) were also obtained.

From these results we have found that the fungus, *Sclerotinia libertiana* is capable of hydroxylating 15β-, 11α- and 11β-positions of steroid nucleus besides 2β-position.

In these experiments, we have prepared two kinds of new 2β-hydroxyl steroid compounds, (I) and (II), with a strain of *Sclerotinia libertiana*.

We are greatly indebted to Dr. K. Tsuda, Dr. T. Asai, Professors of the University of Tokyo and Mr. M. Matsui, Director of this Laboratory for their helpful advice and constant guidance throughout this work.

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On the New Peptide-Type Ergot Alkaloids, Ergosecaline and Ergosecalinine

Sir:

Formerly, H. Rassbach et al.[1] reported that they isolated ergometrine from the saprophytic culture of *Claviceps purpurea* Tul. At about the same time, we[2] also reported on the formation of ergometrine and some other kinds of alkaloids in the saprophytic culture of a *Claviceps purpurea* strain obtained from the Spanish ergot of rye. We succeeded in isolating the following alkaloids in crystalline form from the mycelium (750 g., based on dry weight) and the culture filtrate (45 l.): peptide-type alkaloids (ca. 1.3 g. yield), ergometrine (45 mg.), ergometrinine (96 mg.), agroclavine (32 mg,) and secaclavine

1) H. Rassbach, K. G. Büchel and H. Rochelmeyer, Arzneimittel Forschung, **6**, 690 (1956).
2) M. Abe, T. Yamano, S. Yamatodani, Y. Kōzu, M. Kusumoto, H. Komatsu and S. Yamada, Ann. Rep. Develop. Res. (Agri.) Minist. Educ., 59 (1956).

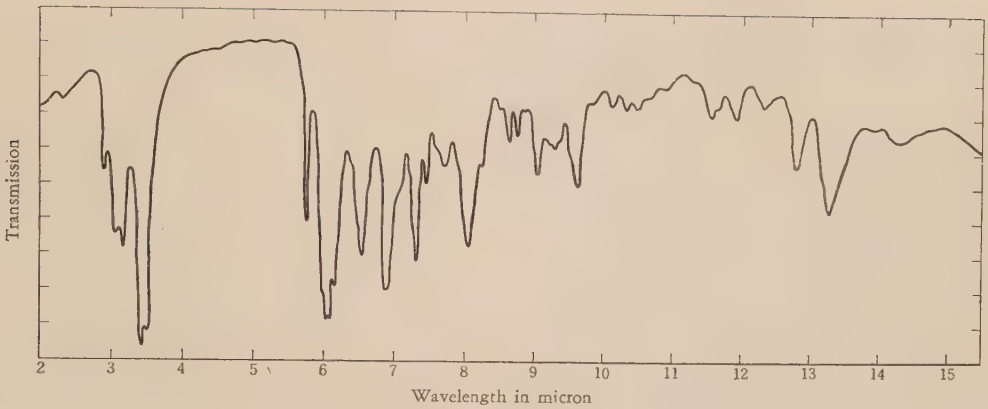


FIG. 1. IR.-Spectrum of Ergosecalinine in Nujol Mull

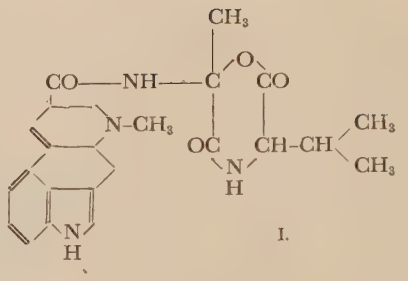
(5 mg.), and further a new water-soluble alkaloid (20 mg.). In this case, the fungus was grown at 24°C for 40 days in 100 flat-bottomed flasks (3 l. capacity) containing each 500 ml. of the medium composed of mannitol (50 g.), KH_2PO_4 (1 g.), $MgSO_4 \cdot 7H_2O$ (0.3 g.), glutamic acid (10 g.), NH_4OH (to pH 5.2) and tap-water (1 l.). The new water soluble alkaloid was isolated together with other alkaloids, also, from the said Spanish ergot itself in almost the same yield.

Presuming that the new alkaloid must be a sort of peptide-type alkaloid having isolysergic acid nucleus in its molecule, we named it ergosecalinine. After that, we[3] found the existence of another unknown water-soluble alkaloid, which was interconvertible with ergosecalinine, both in the ergot and in saprophytic culture of the fungus mentioned, by means of paper partition chromatography; when ergosecalinine and the newly found unknown alkaloid were developed on Toyo filter paper No. 51 with water-saturated ethyl acetate at room temperature, they gave spots with R_F 0.75–0.81 and 0.34–0.36, respectively. In the hope of obtaining this unkown alkaloid, which was probably ergosecaline, in pure state, we have since carried out the extraction and conversion experiments. However, the result was unsatisfactory owing to the small material, but, instead, the chemical properties of ergosecalinine were made clear to some extent by these experiments.

Ergosecalinine was crystallized from ethyl acetate in prisms; m.p. 217° (uncorr. decomp.), $[\alpha]_D^{18} = +298°$, $[\alpha]_{5461}^{18} = +375°$ (c. 0.2 in $CHCl_3$), $[\alpha]_D^{18} = +417°$, $[\alpha]_{5461}^{18} = +512°$ (c. 0.22 in pyridine). *Anal.* Found: C, 66.09; H, 6.68; N, 12.52%; M. W., 450 (by Barger's method). Calcd. for $C_{24}H_{28}O_4N_4$: C, 66.03; H, 6.47; N, 12.84%; M. W., 436.5. It gave a deep blue color with Allport and Cocking's reagent. The UV.-spectrum of ergosecalinine agreed well with those of lysergic and isolysergic acids. The IR.-spectrum of this alkaloid was as shown in Fig. 1. It was insoluble in petroleum ether, sparingly soluble in benzene and chloroform, moderately soluble in ethyl acetate, but readily soluble in acetone, methanol, ethanol and pyridine. Its solutions in organic solvents exhibited a marked fluorescence. It dissolved slightly in water, and readily in dilute acids. Ergosecalinine yielded pyruvic acid together with lysergic acid on alkaline hydrolysis, while, on acid hydrolysis it afforded only one amino acid which was identical with valine. When boiled in a mixture of ethanol and acetone containing phosphoric acid, ergosecalinine was converted into an unknown substance which was more soluble in water than the original one, and which, in turn, was reconverted into ergosecalinine when boiled

3) M. Abe, Y. Tamano, S. Yamatodani, Y. Kōzu, M. Kusumoto, H. Komatsu and S. Yamada, Report at the Annual Meeting of Agr. Chem. Soc. Japan held at the University of Tokyo (April 10, 1957).

with methanol. From this fact and from the result obtained in a degradation experiment, it was evident that this unknown substance was nothing but the alkaloid, ergosecaline, which we were pursueing. As mentioned above, however, this alkaloid has not been obtained in pure state. It was assumed that ergosecaline and ergosecalinine should have a common structure representable by formula I.



I.

By the way, we have recently found that the alkaloid, chanoclavine, reported by H. Hofmann et al.[4] is identical not only in paper chromatographic behavior but also in IR.-spectrum with our secaclavine (alkaloid X[5]), and, therefore, it may not be a new substance.

This work was financially supported in part by a Research Grant from the Ministry of Education.

4) A. Hofmann, R. Brunner, H. Kobel and A. Brack, *Helv. Chim. Acta*, **40**, 1358 (1957).
5) M. Abe and S. Yamatodani, *J. Agr. Chem. Soc. Japan*, **28**, 501 (1954).

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Sulfur-containing Amino Acids in the Embryonated Chicken Egg. (p. *155~159*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

The contents of embryonated White Leghorn eggs were coagulated by boiling in water for 10 minutes and fractionated by the appearance of the heat coagulated materials. In this manner the author could observe soft coagulated yolk and white, hard coagulated yolk and white, not coagulated fraction, embryo and tissue surrounding the embryo. Total sulfur, methionine and cystine of these fractions were determined at 120, 210, 330 and 400 hours of incubation. Yolk and white were partially liquefied during 120~210 hour period and these liquefied parts were softly coagulated by boiling. During incubation, these parts might be readily utilized by embryo. Methionine of white decreased and cystine of it increased with hours of incubation, but these amino acids were main body of sulfur compounds of yolk and white through the all period of incubation. During 210~330 hour period, methionine and cystine were mainly supplied by white to embryo and these amino acids of yolk were almost not utilized by embryo before 330 hours of incubation. In embryo and not heat-coagulated fraction, sulfur compounds other than the sulfur-containing amino acid were observed quantitatively especially during 330~400 hour period. The author identified that the not heat-coagulated fraction was mainly composed of allantoic and amniotic fluid except heat-coagulable proteins of these fluids.

Methionine and Cystine of Abnormal Chicken Eggs. (p. *159~163*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

The purpose of this study is to present information concerning the magnitude of variations found in percentage of sulfur-containing amino acids of yolk and white in the case of abnormal eggs. The abnormal eggs provided here eggs laid by White Leghorn pullets and dwarf and double yolk eggs laid by the same hens whose age ranged from pullets to 2 year olds. Methionine and total nitrogen of white remained fairly uniform during 90~180 day period of the first laying year. Greater variations were observed in the percentage of these components during the first 60 days than occurred in subsequent productions. Methionine of white decreased slightly after the pullets reached 180 days in egg production. The magnitude of variations found in data concerned with yolk were less than these concerned with white through the all first laying year. The components of dwarf or double yolk eggs were not different from these of normal eggs. The author concluded that eggs laid during 90~180 day period were the most suitable to the samples provided for study of changes of these components during incubation or storage etc..

Studies on the Utilization of Ion Exchange Process in Sugar Refining. Part VII. The Relation between Restorating Procedures and Behaviour of Inorganic Substances on the Strongly Basic Anion Exchange Resins from Sugar Solution. (p. *163~166*)

By Susumu IWASHINA

(*Research Institute of Meiji Sugar Mfg. Co. Ltd., Kawasaki, Japan*)

It is known that the anion exchange resin resistance property against oxidizing agents is influenced by small amount of co-existing Cu, Mg, Mn, Fe, Co, or other metals, and that the destruction of chemical and physical function of the resin is promoted.

The accumulation of inorganic components was definitely observed in the contaminated of the resin. When their distribution was qualitatively examined by spectroscopic analysis, the following was found: These metals can less favorably be removed by the NaClO treatment than by the HCl treatment.

As previously reported*, the consequent decrease in the resin power unfavorably affected the retention of the life. The quantitative analysis has further shown that in the HCl treatment the Fe content decreased to 54.1% of the time of the contamination.

From the above, it has been concluded that suitable choice should be made of the above two methods in relation to the pretreatment of influent liquor to indirectly cope with the color contamination of the restorating procedures.

* S. Iwashina: Part VI. *J. Agr. Chem. Soc. Japan*, **33**, 42 (1959).

Determination of Glucose in Fruits by Micro Hypoiodite Method. (Studies on the Quantitative Determination of Sugars in Fruits. Part II).

(p. *166～170*)

By Kazuo OSODO, Hidejiro KAZUMI, Masayuki KOTAKA and Hanao SHITOMI

(*National Instruction Institute of Rural Industry, Shinjo, Yamagata*)

Willstätter-Schudel method, using oxidation of glucose by sodium hypoiodite in alkali solution, has been modified and micronized for the determination of glucose in fruits which contain fructose always. Transfer 5 ml of a sample solution containing glucose less than 2 mg into a 25 mm diameter test tube with glass stopper. Add 5 ml of N/100 iodine solution and 1 ml of 0.15 M sodium carbonate solution, mix, and after 90 minutes add 2 ml of 0.2 N sulfuric acid. Immediately titrate with N/100 sodium thiosulfate solution. Take the difference of titre with a blank. One ml of N/100 sodium thiosulfate solution is equal to 0.840 mg of glucose.

The error is less than 1%, but in presence of fructose ten times the weight of glucose the error is about 9%, five times about 4.5%. Components in fruits adsorbed by cation exchange resin, Amberlite IR-120, give much errors. These substances must be removed on the determination of glucose by this method.

On the Determination of Neutral Amino Acids by Partition Chromatography on Silica Gel.

(p. *170～174*)

By Makoto KANDATSU and Hiroshi NAITO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Although partition chromatography of amino acids on silica gel column, originally introduced by Martin and Synge, has not been put to practical use, it seems necessary to reexamine the conditions which would suggest the convenient way for estimation of mono amino acids.

The authors examined the partition method whether it could apply to protein hydrolysate especially high in methionine content. The procedure was essentially the same as that of Tristram except the oxidative treatment of the protein hydrolysate which effects the separation of valine and methionine fractions, converting the latter to methionine sulfone.

As for the quality of silica gel, which was prepared from the pure products, water content particularly the "structural water" of the gel, (i.e. weight loss of the gel by heating up to 600°,) seemed to affect significantly the amino acid value.

Acetylated amino acid mixture was eluted with ethyl acetate, then developed by chloroform-*n*-butanol mixture in which the latter content was increased from 3 to 10% in the course of the chromatography. The recovery of amino acid was relatively constant (93–105%) except that of tyrosine in the oxidized mixture.

The partition chromatography was applied to the determination of several amino acids in casein and skeletal muscular protein of albino rat and it was shown that the amino acids values obtained were generally acceptable though the values of alanine, proline, tyrosine and methionine were variable.

The estimated value of valine and leucine isomers (leucine plus isoleucine) agreed with those in literatures.

Studies on Flavonoids. Part I. On the Aluminium Salts of Flavonols.

(p. *174～176*)

By Keizo HAYASHIYA

(*School of Textile Fibers, Kyoto Technical University*)

A complex of morin and aluminium of the probable type $Al(morin)_3$ was prepared. A solution of morin in pyridin containing aluminium chloride ($Al_2Cl_6 \cdot 12 H_2O$) was heated for 2 hrs. After isolation in the usual manner followed by pouring the reaction mixture into ice water yellow solid was purified from alcohol (Found: Al, 2.72. Cald. for $Al(C_{15}O_7H_8)_3$: Al, 2.91%).

This complex exhibited an intense fluorescence at pH 4.0. The fluorescence spectrum of this complex showed a transmission band maximum at 495 in acidic alcohol solution and the intensity of this fluorescence is very weak at pH 6～7 or at lower than pH 3.0. The author suggested that the complex which was an intensely fluorescent was not that of the type $Al(morin)_3$.

Studies on Flavonoids. Part II. On the Method of Separation and Purification of Flavonols.

(p. *176～178*)

By Keizo HAYASHIYA and Tsuneo KATO

(*School of Textile Fibers, Kyoto Technical University*)

A method, in which alumina and dilute acid were used as an adsorbent and an eluent respectively, was studied in an attempt to isolate or purify a series of flavonols. For example, quercetin was almost completely adsorbed with alumina and 87 per cent of this pigment was eluted from the alumina with acidic (about N/10) methanol. The practical method was also given on the isolation of flavonols from the greenish cocoon of silkworm or the mulberry leaves. The author sug-

gested that these flavonols were adsorbed on alumina in the type of chlate complex with aluminium.

Biochemical Studies on the Blast Disease of Rice Plant. Part XII. The Isolation of Coumarin from the Blast Diseased Rice Plant. (p. *178~180*)

By Kinjiro TAMARI and Jun KAJI

(*Faculty of Agriculture, Niigata University*)

The authors have isolated coumarin from the stunted rice plant caused by the blast disease, finding no other coumarin-substances such as umbelliferone and scopoletin. Coumarin could not be isolated from the culture liquid of the blast mould, *Piricularia oryzae* Cavara. These facts indicate that coumarin must be formed by the stunted rice plant through its abnormal metabolism. In addition to two toxins, piricularin and α-picolinic acid, excreted by the blast mould, coumarin should take part in the stunting process of the blast-diseased rice plant.

Biochemical Studies on the Blast Disease of Rice Plant. Part XIII. The Accumulation of Coumarin in the Stunted Rice Plant caused by the Ill-Effect of Piricularin. (p. *181~183*)

By Kinjiro TAMARI and Jun KAJI

(*Faculty of Agriculture, Niigara University*)

In the previous paper the authors reported on the coumarin-accumulation in the stunted rice plant caused by the blast disease.

The present communication deals with the coumarin formation in the rice plant, stunted by the severe ill-effect of piricularin, one of the toxins, excreted by the blast mould and with the estimation of the contents of coumarin in the stunted rice plant.

On the Synthesis of α- and β-Lactose-1-phosphate. (p. *183~186*)

By Rinjiro SASAKI and Kôkichi TANIGUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The authors[1] have found that β-galactosidase acts more actively on α-lactose than on β-lactose in limited conditions, in which the interconversions between α- and β-lactose can be disregarded. In order to perform the experiment in biochemical conditions in which the interconversion velocity is very high, the two anomeric isomers must be more stable. 1-Phosphate esters of α- and β-lactose, which have been postulated as an intermediate in lactose synthesis in mammary gland, are more stable and do not undergo isomerizations.

Attempts to synthesize α- and β-lactose-1-phosphate are made in this paper. The α-isomer is prepared by reacting the acetobromolactose with silver diphenyl phosphate and β-lactose isomer with silver dibenzyl phosphate. The specific rotations of prepared α- and β-lactose-1-phosphate are $+73.3°$ and $24.8°$ as Ba salt, and observed hydrolysis constants of them at 37° in 1 N acid are 0.0017 and 0.0057, respectively.

(1) R. Sasaki and K. Taniguchi, *J. Agr. Chem. Soc. Japan*, **33**, 111 (1959).

Chemical Studies on the Autolysis of Meats. Part XII. On the Changes of Free Amino Acids in Meats during Ageing. (p. *186~189*)

By Rinjiro SASAKI, Masao FUJIMAKI and Takehiko KAWANO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

Free amino acids in meats immediately after the slaughter of animals and during ageing of meats were detected by paper partition chromatography. Results obtained are as follows.

(1) Free amino acids in meats (pork, chicken, carp, bonito and tunny meats) immediately after the slaughter of animals were glycine, alanine and glutamic acid (except carp meat).

(2) The contents and kinds of free amino acids in meats seemed to be more in pork, bonito and tunny meat than in chicken and carp meat.

(3) Free amino acids increased during ageing of meats, and amino acids of threonine, leucine, isoleucine and phenylalanine which were not found immediately after the slaughter, occurred during ageing.

Chemical Studies on the Autolysis of Meats. Part XIII. On yellow Coloured Spot by Ninhydrin in the Two Dimentional Paper Partition Chromatography of Meat Extracts. (p. *190~193*)

By Masao FUJIMAKI, Nobuhiko ARAKAWA and Miyoko WATANABE

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

The spot with fluorescence under ultraviolet light, and yellow colour by ninhydrin treatment, in the two dimentional paper chromatogram of alcohol extract of tunny fish meat (Germo macropterus) was found to be consist of two parts.

The one, dialyzable through cellophane tube against redistillated water, was considered to be potassium phosphate (dipotassium salt), and the other seemed to be a peptide which was hydrolysed into aspartic,

glutamic acid, serine, glycine, alanine, lysine, and histidine.

During the concentration of the alcohol extract, white crystal occurred, which was identified to be creatine.

Formation of L-Glutamic Acid from α-Ketoglutarate by Mold Transaminase. (p. *193~195*)

By Kimiyo MICHI, Moto ARAI and Yoko HOSODA

(*Laboratory of Food and Nutrition of Japan Women's University, Tokyo*)

The occurrence of aspartic-α-ketoglutaric transaminase in Penicillium and Aspergillus were investigated. A number of strains of *A. tamarii*, *A. oryzae*, *A. awamori*, *A. sojae*, *A. nidulans*, *A. fumigatus*, *A. ochraceus*, *A. tricolor*, *P. vinaceum*, *P. oxalicum*, *P. roqueforti*, *P. citrinum*, *P. islandicum* and *P. corymbiferum* were found to produce the transaminase in cultured bran. The enzyme catalyzes the formation of L-glutamic acid from α-ketoglutarate and L-aspartic acid or L-alanine.

The enzyme was prepared by extracting it from molded bran (*A. oryzae*) with water (1 : 3.5), and then causing precipitation by the use of 0.6 saturation of ammonium sulfate, dialyzing the aqueous extract of the above precipitate against distilled water and the addition of 2% rivanol solution in order to precipitate non-transaminase protein. Next by the removal of the removal with the use of acid clay, the final aqueous solution of the enzyme was obtained. Aspartic acid (2.5 g) and α-ketoglutaric acid (3.7 g) were added to 250 ml of water, and lime was added till pH 8 was obtained and those acids were dissolved. Then the above transaminase preparation (from 200 g of molded bran) was added. After two days of incubation at 37°C, L-glutamic acid-HCl (3 g, 86% of theo.) was obtained from the reaction mixture. Purified L-glutamic acid (1.5 g, 50% of theo.) gave m.p. 210° and $[\alpha]_D^{18}+31.4°$.

On Indisposed Smell of Saké comes from the Purified Main Mash. (Studies on the Putrefaction of Moromi-mash of Saké. Part IV). (p. *195~197*)

By Masakazu YAMADA, Ryūji FUJII and Kiyoshi YOSHIZAWA

(*The Brewing Experimental Station, Tax Administration Agency*)

In making saké we meet two types of putrefaction, one is the putrefaction of main mash named Fuzō and the other that of saké itself named Hiochi. They show the different characteristic odor each other. We will treat the former here.

From 1801 of saké comes from the putrefied main mash the characteristic indisposed odor was isolated and identified as a component of volatile acids, which was further determined as Acetic acid.

The acid may be produced by some hetero type lactic acid bacteria contaminated in earlier stage of mash.

Thus when we add small quantity of Acetic acid to usual saké, we can easily obtain saké having the same indisposed odor as putrefied main mash.

The indisposed odor will be removed when saké from putrefied main mash is treated with some ion exchange resins.

On the Naturally Occurring Inhibitors of *Aspergillus*-protease. Part V. Mode of Action of the Inhibitors and Influence of Group Specific Reagents. (p. *197~202*)

By Kin'ichi MATSUSHIMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

In this communication, the mode of action was discussed from the standpoint of kinetics of reaction regarding the inhibitors toward the fungal protease and the influence of various group specific reagents upon the activities of inhibitors was also demonstrated. Purified inhibitors from potato, barley, broad-bean, and from egg white (ovoinhibitor[(1)]) were used for these purposes. It was found that the enzyme reacts with each inhibitor stoichiometrically and that this reaction is reversible. It was also shown that each inhibitor acts in a non-competitive manner toward the protease in the presence of substrate. The difference of the susceptibility of the inhibitors toward various kinds of group specific reagents seemed to provide suggestive evidence that different chemical groups may be involved for the activity of each inhibitor.

(1) K. Matsushima; *Science*, **127**, 1178 (1958).

Studies on the Chemical Composition and Quality of Silage. Part III. On the Contents of Ammoniacal Nitrogen and Butyric Acid. (p. *202~205*)

By Hiroshi SUTOH and Senji UCHIDA

(*Laboratory of Animal Feeding, Faculty of Agriculture, Okayama University, Japan*)

Samples of silage obtained from dairyfarms as Tsuyama city, Okayama Prefecture in Japan, were statistically analyzed for the purpose of finding the relationship between the butyric acid content and the ammonia nitrogen content of silage.

In each sample the contents of moisture, total nitrogen,

and ammonia nitrogen as well as pH value were determined by the usual method, and organic acids were determined by means of Flieg's method.

The range of contents was respectively, 0.041～0.824 per cent in ammonia nitrogen, and 0～12.7 per cent in butyric acid, on the dry matter basis; and the percentage of ammonia nitrogen to the total nitrogen was 4～49.

A significant positive correlation, $r=+0.592$, was found to exist between the butyric acid content and the ammonia nitrogen content of silage ($P<0.001$) and also a significant positive correlation, $r=+0.621$, was found between the butyric acid content and the ratio of ammonia nitrogen to the total nitrogen ($P<0.001$).

Flieg's evaluation method, that of Gneist and the standards of American Dairy Science Association committee were respectively applied to the analytical data for the appraisal of quality of these samples. The results of appraisal by these methods seemed almost to agree, if not completely.

The Influence of Feed and Feeding upon the Ruminal Gas Formation. Part V. Influence of Carbohydrates. (p. *206～209*)

By Tatsuro MATSUMOTO

(*Laboratory of Animal Nutrition and Feeding, Faculty of Agriculture, Tohoku University*)

A hundred grams of glucose, starch and cellulose were injected into the fisturated rumen of goat, and changes of rumen gas compositions and rumen liquor properties in each case were compared with one another. CO_2/CH_4 ratio slightly increased after glucose injection, suffered no change in the case of starch injection, and slightly decreased after cellulose injection. The concentrations of volatile fatty acids, ammonia nitrogen, reducing- and non-reducing sugars of the rumen liquors at three hours period after injection were as follows:

Injected substance		Glucose	Starch	Cellulose
Volatile fatty acids	mM/*dl*	5.3±1.2	3.2±0.5	2.8±0.4
Ammonia nitrogen	mg/*dl*	16.5±0.5	15.3±0.6	19.6±1.7
Reducing sugars	mg/*dl*	74.0±2.0	75.2±4.9	69.0±3.0
Non-reducing sugars	mg/*dl*	26.0±7.4	25.8±6.8	25.8±4.0

(mean values and standard deviations)

Studies on the Butanol Formers of Saccharotypes. Part I. The Isolation of Two Strains. (p. *210～211*)

By Kazuo HOSHINO, Hidenori ISHIKURA and Kenzo WATANABE

(*Kyowa Fermentation Industry Co. Ltd.*)

Two butanol formers of saccharotypes were isolated from soils and nodules. These strains fermented molarses and the yield of solvents exceeded 28% to fermented sugars. Also they have the strong resistance to bacteriophage. Morphological and physiological studies demonstrated that these strains were close to *Cl. butyricum*.

Studies on the Organism Producing Isopropanol from Acetone. Part I. About *Lactobacillus brevis* var. *hofuensis* nov. var. (p. *212～216*)

By Kazuo HOSHINO

(*Kyowa Fermentation Industry Co. Ltd.*)

From an acetone-butanol fermentation which showed the unanticipated presence of isopropanol a contaminant was isolated.

It was demonstrated that this organism formed isopropanol in the medium which contained acetone as an ingredient and that also about 80% of acetone were reduced in mixed culture with *Cl. acetobutylicum*.

Morphological and physiological tests identified this organism as a lactobacillus of the heterotypes and the name of *Lactobacillus brevis* var. *hofuensis* nov. var. was designated owing to the properties of acetone reduction and sucrose fermentation.

The intact cell of this organism catalysed oxio-rediction between isopropanol and acetone. Furthermore, activities to reduce acetone were compared with other organisms.

Studies on the Chromatography of Starches. Part I. Paper Chromatography of Starches. (p. *216～218*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

Paper chromatography of starches was made with a KOH solution as the developing solvent by the ascending technique. Filter paper strips (Tōyō, No. 2; 2×25 cm) were washed with a 15% KOH solution by the descending technique and air-dried after washing out in running tap-water. This treatment was repeated. 0.003 ml of the starch solution dissolved in 0.5 N KOH (5 mg/ml) was placed on the starting line of the strip and air-dried. The paper was developed with 1 N KOH solution by the ascending technique until the solvent front moved 15 cm from the starting line. The paper was immediately dipped into a 10% acetic acid solution and then stained by dipping in a 0.2% iodine solution in 50% alcohol. The amylose fraction moved about to the center of the chromatogram. The amylopectin fraction remained at the original point. Thus, by this

procedure, starch was separated into two fractions of amylose and amylopectin.

Studies on the Chromatography of Starches. Part II. Quantitative Paper Chromatography of Starches. (p. *218~220*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

Amylose content of starches was determined by the quantitative paper chromatographic technique. A filter paper strip (2×15 cm.), pre-treated with 15% KOH (see, preceding paper), was marked with pencil at a starting line 5 cm. from the top edge of the strip. Starch was dissolved in 0.5 N KOH solution. 0.05 ml. of the solution (10 mg./ml.) was placed in a zone 2 cm. wide on the starting line of the strip. Immediately the paper was developed with 0.8 N KOH solution by the descending technique until the solvent front reached to the bottom edge of the strip. The paper was stained with 0.2% iodine solution in 50% alcohol, after being neutralized with acetic acid solution. It was found that amylopectin fraction remained at the starting line and amylose fraction moved descending-wise in an irregular zone.

For quantitative separation of amylose fraction, the irrigation of the chromatogram with the solvent was run until 2 ml. of the eluate of the amylose fraction was obtained. The eluate was neutralized with acetic acid solution and colored with I-KI solution. The optical density was measured using a photoelectric colorimeter. The concentration of amylose in the solution was determined by referring to a calibration curve of amylose concentration vs. optical density which had been prepared by chromatographing the standard solutions of known amylose concentrations, using the same technique as described above. Amylose contents of potato and ordinary rice starches were determined by this procedure.

Formation of L-Glutamic Acid from γ-Aminobutyric Acid by Bacteria. (p. *221~224*)

By Toshinao TSUNODA, Ryohei AOKI, Kazumoto KINOSHITA and Yasuhiro KONDO

(*The Central Research Laboratory of Ajinomoto Co., Inc.*)

Screening tests for L-glutamic acid (L-GA) producing strains on a medium containing γ-aminobutyric acid (γ ABA) as a chief ingredients were carried out. Many strains of bacteria which are able to accumulate L-GA in the cultural broth were obtained. They were widely distributed among various genera, and two of them showed the excellently high level of L-GA accumulation. Diagnosis of these strains showed that one belongs to *Brevibacterium helvolum* and another belongs to *Bacillus pumilus*. The addition of a small quantity of glucose to a medium resulted in promoted growth and increased yield of L-GA in shake culture. The course of fermentation are shown using the authors strain of *Bacillus pumilus* and the medium initially containing γ ABA, glucose, minerals, etc. The yield of L-GA was 63% of γ ABA consumed.

Biochemical Study of Rice Starch. Part III. Chemical Properties of Rice Starch prepared from Various Kinds. (p. *225~230*)

By Humio KURASAWA, Ikuo IGAUE, Tosiro HAYAKAWA and Hiroshi ÔGAMI

(*Niigata University, Department of Agriculture*)

Various kinds of rice, Norin No. 1, Kosiziwase, Norin No. 36 and Aikoku No. 70 were cooked and the qualities of each were investigated. The boiled rice of Norin No. 1, Kosiziwase and Norin No. 36 was more glutinous than that of Aikoku No. 70. So the author prepared the starch from each variety of rice and examinated the properties of starch.

By using Brabender's amylography, it was found that the swelling temperature was lowest for the starch separated from Aikoku No. 70 rice. The starch from Aikoku No. 70 rice contained 24~25 per cent amylose but another starch from Norin No. 1, Kosiziwase, Norin No. 36 rice contained 20~21 per cent amylose.

Enzymatic and Microbiological Studies on Lactose-l-phosphate. (p. *230~233*)

By Kôkichi TANIGUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo*)

It has been found by the authors[1] the β-galactosidase had greater affinity to α-lactose than to β-lactose. But the interconversions between α- and β-isomers were unavoidable. So in this experiments lactose derivatives, α- and β-lactose-l-phosphate, which did not interconvert each other, were used as substrate. The results of the experiments are as it follows.

i) β-Galactosidase did not act on both α- and β-lactose-l-phosphate, therefore the difference of enzymic action on lactose isomers could not be recognized.

ii) *Esch. coli*, *Aer. aergenes* and lactose fermented yeasts which had both phosphatase and β-galactosidase action, could utilize lactose-l-phosphate as carbon source. But lactic acid bacteriae which were lacking in phosphatase action, could not utilize lactose-l-phosphate.

iii) It became clear that α- and β-lactose-l-phosphate had almost the same effects on the growth and O_2-uptake of *Esch. coli* and *Sacch. fragilis*.

(1) Rinjiro Sasaki and Kôichi Taniguchi, *J. Agr. Chem. Soc. Japan*, **33**, 111 (1959).

Polarographic Studies of Lysozyme. Part I. The Catalytic Protein Wave of Lysozyme in Ammonia Buffer Containing Cobaltous Chloride. (p.*233*~*238*)

By Masahide ITO

(*Chemical Laboratory, Faculty of Fisheries, Prefectural University of Mie*)

In order to elucidate the catalytic protein wave the polarographic behavior of several proteins have been examined. In the present paper the results obtained with lysozyme in ammonia buffer containing cobaltous chloride will be described.

Lysozyme gave a characteristic protein double wave in this medium. The shape of the wave was different from those of the other proteins such as serum albumin, ovalbumin and whole casein, and the wave-height was higher.

The dependence of the wave-height on the concentration of lysozyme, $CoCl_2$, NH_4Cl or NH_3 was, however, similar to the case of other proteins.

The temperature effect characteristic to lysozyme, the height of the first wave increased with temperature, while that of the second almost unchanged, seems to suggest the intimate relationship between the polarographic behavior and its thermal stability, accordingly its specific chemical configuration.

The wave-height has, moreover, been reported to be closely related to the cystin content of protein in general.

So the characteristic behavior of lysozyme mentioned above would be reduced to its chemical configuration, especially its high cystin content, though the other properties, for example, its isoelectric point lying near the pH of the medium etc., should not be neglected.

Polarographic Studies of Lysozyme. Part II. The Catalytic Protein Wave of Lysozyme in Ammonia Buffer Containing Hexamminecobalt (III) Chloride. (p. *238*~*243*)

By Masahide ITO

(*Chemical Laboratory, Faculty of Fisheries, Prefectural University of Mie*)

The catalytic protein wave of lysozyme was further investigated in the medium containing hexamminecobalt (III) chloride.

Lysozyme gave also, in this case, the characteristic protein double wave. The shape of the wave was different from those of other proteins and the wave-height was higher.

The dependence of the wave-height on the concentration of lysozyme, $Co(NH_3)_6Cl_3$, NH_4Cl or NH_3 was, however, similar to the case of other proteins except that on the protein concentration which was characteristic to lysozyme.

Compared to the results mentioned in the preceding paper, in this case, the wave-height was higher and the dependence of the wave-height on the medium constituents or the temperature differed somewhat. The shape of the wave seemed to be not identical, but become similar to each other under certain conditions.

Such difference in behavior of lysozyme caused by two cobalt salts would probably due to the difference in the concentration of $Co(NH_3)_6^{++}$ in the neighborhood of the electrode and the extent of the complex formation between the protein and the cobalt complex ions.

Therefore, the polarographic behavior of lysozyme would depend not only on its specific configuration, its high cystin content etc., but also on the cobalt complex contained in medium.

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Studies on the Chromatography of Starches. Part III. On the Separation and Determination of Amylose of Starches by means of Paper Chromatography. (p. *245~249*)

By Motoji TAKI

(*Faculty of Agriculture, Mie University*)

After a filter paper strip (4×20 cm.) was washed with 15% KOH solution by the descending technique, it was washed in running water and air-dried. This treatment was repeated with 35% $HClO_4$ solution. Four lines, A, B, C and D, were marked with a pencil 3, 9, 11 and 15 cm. from the top edge of a strip, respectively. Starch solution was placed between A and B as a wide zone and air-dried. The chromatogram was developed with 35% $HClO_4$ by the descending technique until the solvent front reached D. Amylopectin fraction remained between A and B. Amylose fraction, contaminated very slightly with amylopectin, descended to the solvent front. The part of the developed chromatogram containing the amylopectin fraction was cut off along line B. When this strip was folded along line C and suspended in a descending chromatographic equipment containing 0.2 N I_2-KI solution, the strip was stained blue along D. Then the strip was irrigated with 35% $HClO_4$ solution. The reddish blue staining amylopectin fraction descended from D and was eluted finally from the strip, but the blue staining amylose fraction remained along D. After 35% $HClO_4$ solution had been washed out from the strip with 0.1% iodine solution in 50% alcohol by the same technique as described above, the strip was air-dried and was allowed to stand until the blue color almost disappeared. The amylose fraction was eluted with 35% $HClO_4$ solution. The amount of amylose in the elute was determined colorimetrically with iodine.

Studies on the Reticulo-rumen Digestion. Part IX. Distribution of Nitrogen and Cobalt in the Rumen under Fasting. (p. *249~254*)

By Makoto KANDATSU, Bunpei MORI and Toshio OZAKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The rumen content gathered through the fistula at the rumen of goat is divided into 6 parts, i. e., coarse fodder residue, infusoria, fine fodder residue, bacteria and filtrate (infusoria and bacteria free fraction) by filtration and centrifuging.

The nitrogen and cobalt contents of each fraction are determined by usual methods titrimetrically and colorimetrically, respectively. The nitrogen content in the coarse fodder fraction decreases with the time course of fasting, but does not decrease in the total filtrate, being almost at the constant level. Among the total filtrate, remarkable change appears in the infusoria and bacteria fractions in such a way that at first the nitrogen content of both fractions decreases, but subsequently infusoria fraction increases after a some while of increase of bacteria fraction and then both fractions decrease almost at the same way.

The cobalt content of each fraction changes as same as the nitrogen in the whole. The number of infusoria per unit volume of total filtrate does not decrease contrary to our expectation under fasting.

It is assumable from our results that cobalt has more important role in the bacteria than in the infusoria among the microbes of the rumen.

Studies on the Reticulo-rumen Digestion. Part XI. On the Concentration of Volatile Fatty Acids in the Rumen. (p. *255~258*)

By Makoto KANDATSU and Fumio KUMENO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Determinations of volatile fatty acids in the rumen of a goat fed on poor meadow hay and concentrate mixture were made. Mean levels of volatile fatty acids increased to peak value of 8~9 m. mol/100 ml. between 4~7 h. after feeding. Then these decreased gradually to the level of 2~3 m. mol/100 ml. 48 h. after feeding.

Variation of the molar percentage of individual volatile fatty acids was studied by partition chromatography. On feeding twice a day, there was a little variation in the molar percentage of individual acids. The range of that of acetic, propionic and butyric acid was respectivery 67~73%, 16~22%, 7~13%.

The same result was gained on feeding once a day. The range of molar percentage of acetic, propionic and

butyric acid was 73~76%, 15~19%, 7~10% respectively.

Reaction of Furan Derivatives with Ammonia. Part II. On the Reaction of 2-Acetylfuran with Ammonia. (p. *259~261*)

By Hiroshi SUGISAWA and Kiyoshi ASO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai*)

It has previously reported that β-hydroxy pyridine derivatives were formed from furfural derivatives and ammonium salts.

One object of the present investigation is to prove a reaction mechanism; the other object is to provide an improved process for the preparation of β-hydroxy pyridines by reacting 2-acetylfuran with ammonia. The authors have found it possible to obtain in good yield 2-methyl-3-pyridol (40–60%) and 2-acetylpyrrole (20–25%) by reacting 2-acetylfuran with ammonia at 180° in several conditions as shown in the following, and also the authors have found that two substances were obtained from 2-acetylfuran with liquid ammonia by using ammonium chloride as a catalyst.

1) 2-Acetylfuran, Liquid ammonia and Ammonium chloride.
2) 2-Acetylfuran, Liquid ammonia and Methanol.
3) 2-Acetylfuran, 28% Aqueous ammonia and Methanol.
4) 2-Acetylfuran, 10% Alcoholic ammonia and Ammonium chloride.

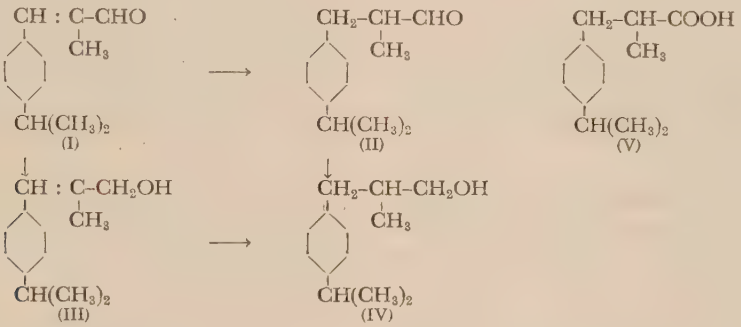
The reactants were heated in an autoclave at 180° for 20 hrs., the product was treated with active carbon. 2-Methyl-3-pyridol and 2-acetylpyrrole were respectively separated by the sublimation at reduced pressure. 2-Methyl-3-pyridol was recrystallized from benzene, colorless prism, m. p. 168°. Its picrate was yellow needle, m. p. 203°. 2-Acetylpyrrole was recrystallized from water, colorless needle, m. p. 91°.

Studies on the Preparation of Cyclamen Aldehyde and Its Related Compounds. Part IV. On the Partial Reduction of *p*-Isopropyl-α-methylcinnamaldehyde. (p. *261~266*)

By Seishi SHIN'YA

(*Mitsuwa Chemical Laboratory, Marumiya Co., Tokyo*)

The most classical method[1] for the preparation of cyclamen aldehyde (*p*-Isopropyl-α-methylhydrocinnamaldehyde) (II) was the partial reduction of *p*-Isopropyl-α-methylcinnamaldehyde (I), on which the by product

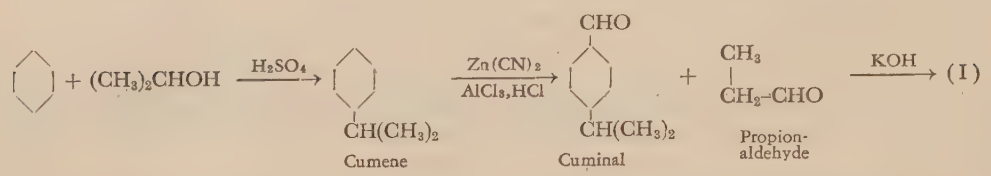


formation of both unsaturated (III) and saturated (IV) alcohol was supposed to arise.

In the present report the author confirmed this supposition to be a fact from the hydrogen-absorbing-aldehyde decreasing curve on the partial reduction of (I), and as well from the vapor-phase chromatograms as from the infrared spectra of the products thus prepared. The contents of these by products (III) and (IV) influenced the purity and grade of the product so much that the author tried to obtain them synthetically and to investigate their physical and odorous properties in their pure states.

At first the author prepared the pure cumene (isopropylbenzene) (b. p., 152~3°C; n_D^{30} 1.4947) by the condensation of isopropylalcohol and benzene in the presence of sulfuric acid (ca. 80%)[2], and then converted it to cuminal (b. p. 82~4°C/3.5 mm Hg; n_D^{20}, 1.5301) by modified Gatterman Koch reaction[3]. Cuminal was condensed with propionaldehyde[4] to yield (I) (b. p. 154~9°C/10–11 mm Hg; n_D^{30}, 1.5810).

Additionally pure (III) (b. p. 135~8°C; n_D^{20}, 15597; Phenylurethan, m. p., 76°C) was obtained by the action of sodiumborohydride $NaBH_4$ on (I) and (III) was hydrogenated to (IV) (b. p., 130°C/7 mm Hg; n_D^{20}, 1.5110) in the presence of Raney nickel catalizer. The



infrared spectra of (I) (III) and (IV) were cited in this paper together with those of pure (II) (b. p., 112~4°C/4 mm Hg; n_D^{20}, 1.5050) and *p*-Isopropyl-α-methylhydrocinnamic acid (b. p., 151~2°C/3 mm N. V., 270.35) (V). The preparation of the latter materials was reported in the previous papers[5,6].

1) A. Knorr, A. Weisenborn and Winthrop Chem. Co., U. S. P. 1,844, 013/1929.
2) H. Meyer, K. Bernhaner; *Monatsch.*, **53/54**, 721 (1929).
3) R. Adams, E. Montogomery; *J. A. C. S.*, **46**, 1518 (1924).
4) C. D. Hurd and B. N. Meinert: *Org. Synth.*, **12**, 64 (1932).
5) S. Shinya; *J. Agr. Chem. Soc. Japan*, **29**, 96 (1955).
6) S. Shinya; *ibid.*, **29**, 93 (1955).

On the Oxidative Fermentation. Part XVII. α-Ketoglutaric Acid Fermentation. (3) (p. *266~271*)

By Toshinobu ASAI, Katsumi SHIMZU and Haruyoshi MUROOKA

(*Institute of Applied Microbiology, University of Tokyo*)

The chemical pathway of α-ketoglutaric acid formation from glucose was searched with *Kluyvera citrophila* α, a new bacterial species producing a great amount of this acid. α-Ketoglutaric acid was produced from glucose, gluconic and 2-ketogluconic acids by the washed cells of this microorganism and further from pyruvic acid. Since pyruvic acid was also detected in the reaction mixture, it was supposed that pyruvic acid is an intermediate in the course of α-ketoglutaric acid formation from glucose and that the direct oxidative pathway comprising glucose ⟶ gluconic acid ⟶ 2-ketogluconic acid ⇢ pyruvic acid should be present in this reaction, although it is not certain that this oxidation path is a main route of pyruvic acid formation.

Each member of TCA cycle was metabolized with O_2 uptake and CO_2 evolution by the washed cells or cell-free extracts. Moreover, it was ascertained that α-ketoglutaric acid was produced from each member of TCA cycle.

From these results, it was mightily demonstrated that TCA cycle presents in the oxidative pathway of glucose metabolism by this microorganism and that α-ketoglutaric acid might be produced from pyruvic acid via TCA cycle and accumulated by the reason of hardness of its oxidative decarboxylation to succinic acid.

On the Oxidative Fermentation. Part XVIII. α-Ketoglutaric Acid Fermentation. (4) (p. *271~274*)

By Toshinobu ASAI, Katsumi SHIMIZU and Haruyoshi MUROOKA

(*Institute of Applied Microbiology, University of Tokyo*)

In our previous paper, the chemical pathway of α-ketoglutaric acid formation from glucose by *Kluyvera citrophila* α was discussed and it was supported that this acid is produced through the path comprising glucose ⇢ pyruvic acid ⟶ TCA cycle ⟶ α-ketoglutaric acid. In this report the reality of the presence of this pathway was examined. If pyruvic acid is metabolized via TCA cycle, CO_2 fixation of pyruvic acid to form oxalacetic acid should be present. It was ascertained by the radioautography method that α-ketoglutaric acid produced aerobically from glucose or pyruvic acid in the presence of $NaHC^{14}O_3$ was radioactive. Therefore, it is clear that CO_2 fixation reaction is included in the pathway of α-ketoglutaric acid formation from pyruvic acid. Moreover, it was recognized by the degradation experiment with the product obtained that radioactive carbon of α-ketoglutaric acid was mainly located at the α-carboxy radical of this compound.

This result was coincident with the assumed chemical pathway of α-ketoglutaric acid formation by this microorganism presented in the previous paper.

Studies on the Rices Cultured by the Eary and Late Season Growing. Part I. Physical and Chemical Properties of Non-glutinous Rices and their Starches. (p. *275~280*)

By Hiroshi SUZUKI, Shinjiro CHIKUBU and Tatsuo TANI

(*Food Research Institute, Ministry of Agriculture and Forestry; Kagawa Agricultural Experiment Station*)

Three varieties of *japonica* type rice were cultured by two different methods of early and late season growing. Milled late season rices (I) were easily gelatinized by 1.7% KOH, while milled early season rices (II) were hardly gelatinized. The cooking quality of the both rices was compared, the residual solutions of (II) had higher blue value and more dry matter than (I). The starches from (II) had higher blue value and much

higher visco-elasticity than the starches form (I). According to the alkali-viscograms of both starches, starches from (I) were more resistant to gelatinization by KOH than the starches from (II).

Studies on the Chemical Constituents of Tea Leaves. Part IX. The Sugars in Tea.

(p. *280*～*281*)

By Takasi MATSUMURA and Yajiro SAKATO

(*Research Institute of Tea Industry Kyoto, Kyoto Pref.*)

An aqueous extract of manufactured tea after removal of polyphenolic substances, pigments, caffeine and passage through ion-exchange resines was examined for sugars. Glucose, fructose, sucrose, raffinose and one oligosaccharide were detected by paper chromatography. Sucrose and raffinose were isolated as Sr-salt. The content of sucrose is 1.8～2.4% in the ordinary tea and 0.5～1.5% in the shaded tea and raffinose is less than 10 mg%.

Studies on the Change of Chlorophyll by Heating. Part I. On Decomposition Conditions of Chlorophyll.

(p. *281*～*285*)

By Osamu IGARASHI, Kenzo KOSUGE and Yoshito SAKURAI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Between fresh tea leaves and tea products (especially, Sencha) there existed differences in their spectra of acetone extractive fractions. The spectrum of the former had maxima absorption at 430, 660 mμ and the latter at 410, 450, 665 mμ of wave length. As the same differences were found between raw and heated tea leaves, it was supposed that some decomposition of chlorophyll had occured by heating. But, in spinach leaves, the change of spectrum was not recognized by heating.

It was found that the rate of decomposition of chlorophyll was influenced mainly by the pH and the co-existing anions of the leaves or the solution of chlorophyll.

When chlorophyll a separated and purified from spinach leaves was heated in pH 6.0 phosphate buffer, the same changes of spinach appeared, but not in pH 7.0. When the same treatment was carried out with trismaleite buffers, the changes occured not only at pH 6.0 but also at pH 7.0. While, chlorophyll b was scarcely changed by the same heating.

Isolation of Starch Granules from Sweet Potato by Decomposing its Tissues with Bacteria. Part I. On the Strain.

(p. *286*～*289*)

By Motoyoshi HONGO and Toshiro KURUSU

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

The crude culture breaking down a piece (about (1.0–1.5 cm)^3) of raw sweet potato has been said to grow only in the medium which contained some pieces of raw sweet potato in sterile tap water. So it has been difficult to isolate the pure culture. Using a mixture of potato juice agar and Difco's brewer anaerobic agar and incubating in the anaerobic condition at 33°, this strain was purely isolated. The pure culture broke down completely the pieces of raw sweet potato in 10 hrs. It was identified to be *Clostridium acetobutylicum* McCoy, Fred, Peterson, Hastings. In the fermentation of glucose (5.7%) bouillon, sucrose (5.8%)·soy bean cake (0.3%)·$(NH_4)_2SO_4$(0.3%) mash, or molasses (inverted sugar 6.2%)·soy bean cake. (0.3%)·$(NH_4)_2SO_4$ (0.3%) mash, the yield of solvents was 34–35%, and acetone: butanol: ethanol was 38–42: 50–53: 5–9. Other 30 cultures of *Cl. acetobutylicum* had no ability to break down the piece of raw sweet potato. The crude culture has been preserved in ampule after incubating in the sterile water containing pieces of raw sweet potato, but the pure culture was preserved in the sand culture, so the preservation was very simplified.

Studies on *Euglena viridis* as the Nutrient Sources. Part I. On the artificial Culture of *Euglena*.

(p. *290*～*293*)

By Ooki NAKAYAMA*, Sachiko SAKAI*, Seiji TADA*, Bunzo ROKUSHO** and Shintaro KAMIYA**

(**Laboratory of Agricultural Chemistry, Faculty of Agriculture, Tamagawa University, Tokyo; **Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka*)

Chlorella has been studied for many years as the food of tomorrow. The authors tried to culture and analyse the green flagellata Euglena, and they suggested that Euglena is a prosperous nutrient resource.

Eight strains of *Euglena viridis* were isolated trading on the phototaxis of the organism, proper condition of mix-culture with bacteria is determined, and outdoor culture was tried.

Euglena propagates not only in the lights, but also in darkness, utilizing organic acids produced by bacteria from organic waste materials, or eating bacterial cells.

Englena is easily harvested by scooping viscous perricle spreading on the water surface. In the out-door culture, 138 g of dried sample was obtained from 1 m^2 of water

surface within 20 days.

Analytical data will be cited on Part II.

Studies on *Euglena viridis* as the Nutrient Source. Part II. On the Amino Acids Composition and Contents of Vitamins in *Euglena viridis*.

(p. *293～295*)

By Bunzo ROKUSHO*, Shintaro KAMIYA*, Seiji TADA**, Ooki NAKAYAMA** and Sachiko SAKAI**

(**Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University; **Department of Agricultural Chemistry, Faculty of Agriculture, Tamagawa University*)

To know the value of *Euglena viridis* as food-stuff, the general composition, the quality of the protein, and vitamins were studied. It was found that the dry matter of it contained about 54 percent of crude protein, and about 19 percent of crude fat. The contents of 18 amino acids of the protein were estimated by microbiological method, and 10 vitamins were analyzed by chemical or microbiological method.

In vitamins examined the amount of vitamin A, B_1, B_{12}, biotin, pantothenic acid were found rich, and it is notable that B_{12} content (366 mγ/g) was almost equal to that of cattle's or domestic fowl's livers.

From the results of analyses it seems that the nutritional constituents of *Euglena viridis* is resembled to that of animal food-staffs.

Studies on Legume Starches. Part IV. Separation and Determination of Amylose and Amylopectin. (2) Iodine Coloration and Potentiometric Titration.

(p. *296～300*)

By Shin'itirô KAWAMURA and Minoru TADA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kagawa University, Mikityô, Kagawa-ken*)

Amylose and amylopectin were separated from 8 legume starches and potato starch by the use of butanol following complete dispersion in 1% sodium hydroxide (some legume starches were found to be dispersed in this alkali solution only after longer standing than potato starch). Measurements were made of blue value of iodine coloration as well as of iodine affinity by potentiometric titration on the starch, amylose, and amylopectin. Calculation by the latter method gave the following amylose content of each starch: potato 21, broad bean 27, adzuki bean 25, mung bean 29, common bean (2 varieties) 29 and 30, *Vigna sesquipedalis* (2 varieties) 24 and 25, *Dolichos lablab* 23, and sword bean 22%. The starches and the two fractions from these legumes were examined for their intrinsic viscosity and alkali lability number.

Studies on Legume Starches. Part VII. Summarizing Discussion.

(p. *300～304*)

By Shin'itirô KAWAMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kagawa University, Mikityô, Kagawa-ken*)

Legume starches were classified into two groups from the Brabender amylogram: group A (hot-paste viscosity is low) and group B (hot-paste viscosity is high). The starches from legumes of group A (broad beans, peas, and common beans) had larger granules and higher amylose content (27–29%), while the starches from legumes of group B (adzuki beans, *Vigna sesquipedalis*, *Dolichos lablab*, and *Canavalia gladiata*) had smaller granules (exception: *Canavalia*) and lower amylose content (22–25%) Mung-bean starch had smaller granules and belonged to group B, though amylose content was high (29%). Starch from *Stizolobium hassjoo* had smaller granules and belonged to group B. Discussion was made also in connection with botanical classification of the legumes studied.

The Secretion of Nucleotides by Yeast Cells. Part I. Influences of Physical and Chemical Factors on the Secretion by Yeast Cell Suspention.

(p. *304～308*)

By Masataka HIGUCHI and Teijiro UEMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

When the yeast cells were incubated in a suitable medium, such as 0.08 M Na-citrate buffer containing 2% glucose, the ultra-violet (UV) absorbing materials were secreted into the medium from the yeast cells. This activity was comparatively higher in the beer yeast and lower in the baker's yeast than other yeast strains.

The secreted materials of the beer yeast cells, showed the absorption spectrum which has maximum at 258 mμ and minimum at 240 mμ. A large part of them is probably nucleotides such as AMP, CMP etc. The rate of secretion was reduced to an extent of 30 or 40 per cent by the incubation in distilled water instead of the Na-citrate buffer, and about 70 per cent by K replacing Na in the citrate buffer. Inhibition of the activity was not observed in the presence of usual antibiotics and antimetabolites, except a little depression by monoiodoacetic acid.

It is very interesting to note that good nutritional conditions for the yeast growth were unfavourable for

the secretion of the UV absorbing materials by the yeast cells, while on the contrary, the citrate ion and EDTA, a kind of chelating agents were strongly stimulative for it.

Synthesis of Disaccharides by Mercuric Salt. Part I. Synthesis of 3-O-α-D-Glucopyranosyl-D-glucose (sakebiose or nigerose) (I). Reaction of 1, 2-5, 6-Di-O-isopropylidene-D-glucofuranose and 2, 3, 4, 6-Tetra-O-acetyl-α-D-glucopyranosyl bromide. (p. *309~313*)

By Kazuo MATSUDA and Takeshi SEKIGUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

In order to obtain 1, 3-α-linked diglucose (sakebiose or nigerose), 1,2-5,6-di-O-isopropylidene-D-glucofuranose and 2, 3, 4, 6-tetra-O-acetyl-α-D-glucopyranosyl bromide were condensed in dry benzene in the presence of mercuric cyanide, and the reaction products, after removal of the protecting groups, were fractionated by Carbon : Celite column chromatography. Besides 1,3-linked laminaribiose and sakebiose, 1,6-linked gentiobiose and isomaltose were obtained which were characterised as their crystalline octaacetates.

Butanol Fermentation. Part XXX. Variant Strain of *Clostridium acetobutylicum* 314 (Weizmann Type). (p. *313~319*)

By Motoyoshi HONGO*, Ken NAGATA*, Ryōzō HARADA**, Kyosuke AKABOSHI** and Yukio NISHIMURA**

(**Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University; **Yatsushiro-Factory of Sanraku Shuzō Co.*)

In the acetone-butanol fermentation of dried sweet potato by *Clostridium acetobutylicum* 314, an abnormal fermentation happened in which the titrable acidity did not drop from the peak and the evolution of gas ceased. After several days the fermentation progressed again. It was like a bacteriophage attack. According to the usual technique of selecting immune strains, a strain was isolated by plating out the subculture incubated with the addition of small amounts of bacteria-free filtrate of abnormally fermenting mash and selecting colonies. This strain was resistant to the filtrate and did not form 'head' in the culture and formed few spores. Its cells were filamentous in the beginning of growth. These characters are generally those of immune strains. On the other hand, this strain did not ferment trehalose and melezitose, while the original strain did. Although the fermentation is generally slower and the solvent yield is slightly less in the case of immune strain, this strain had a stronger ability to ferment sweet potato and sugar than the original strain. In the fermentation of dried sweet potato (6.0–6.5% as inverted sugar) the fermentation period was 1.5 days and the yield was 33–35%, while those of the original strain were respectively 2.5–3.0 days and 30–32%.

Biosynthsis of Aromatic Compounds by *Bacillus*. (1) (p. *319~324*)

By Hisao ISHIKAWA and Tae OKI

(*Faculty of Agriculture, Ehime University*)

For studing on the mechanism by which plants accomplish the feat of synthesizing lignin from shikimic acid, the biosynthesis of aromatic compounds by *Bacillus* was investigated.

Bacillus subtilis, *Bacillus cereus* and *Bacillus megatherium* were incubated in the Hyduck's medium contained shikimic acid, quinic acid or the aromatic compounds, and the accumulated compounds in their culture filtrates were isolated and identified.

It was established by this method that shikimic acid, quinic acid and 5-dehydroshikimic acid are key intermediates in the biosynthesis of protocatechuic acid or gallic acid in these *Bacillus*, and that protocatechuic acid is a precursor in gallic acid biosynthesis.

Antibiotic Production by Spore-forming Bacteria. Part III. Bacitracin Formation by the Protoplasts of *Bacillus subtilis* st. Tracy. (p. *325~329*)

By Tokujiro AIDA

(*Laboratory of Agricultural Microbiology, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

Protoplasts of the bacitracin producer (*Bacillus subtilis* st. Tracy) were prepared by the lysozyme treatment in hypertonic solution. The protoplasts obtained from the unstarved cells secreted a small amount of bacitracin in buffer solution. No bacitracin, however, secreted by the protoplasts from the starved cells in buffer solution, nor released in the burst preparation shocked osmotically. Using such preparations, 7.9 U/ml of bacitracin-like substance(s) were formed by each mg N of the preparation under the anaerobic condition in the complete system contained amino acid mixture and energy sources (HDP and ATP).

Ultraviolet absorption of the bacitracin-active substance(s) formed by the protoplasts exhibited a broad maximum at about 254 mμ corresponding to bacitracin A, B, D and E, additional unknown shoulder at 280 mμ. Further, the same sample showed six antibacterial spots

on the bioautogram. The highest R_F spot was similar to the standard commercial bacitracin and the other lower five spots were unknown which were named as bacitracin X group.

Antibiotic Production by Spore-forming Bacteria. Part IV. Bacitracin Secretion by the Cell Suspensions of *Bacillus subtilis* st. Tracy. (p. *329~333*)

By Tokujiro AIDA

(*Laboratory of Agricultural Microbiology, Faculty of Agriculture, Tohoku University, Sendai, Japan*)

Unstarved cells of the bacitracin producer (*B. subtilis* st. Tracy) secreted a large amount of bacitracin by the incubation with buffer solution. While the starved cells secreted bacitracin anaerobically in the presence of both glucose and amino acid mixture, and aerobically markedly in the presence of amino acid mixture alone. The bacitracin-active substance(s), secreted by the starved cells incubated under the aerobic and anaerobic conditions, gave the one same spot equal to the standard commercial bacitracin on the bioautogram. The broth filtrates of the organism at the various growth phases showed the same antibacterial spot.

To define the bacitracin-active substance(s) pooled within the cells, the sonickate of the cells was adjusted to pH 4.2, and the resulting precipitate and supernatant were bioautographed. The supernatant showed the one spot equal to the standard commercial bacitracin and the precipitate some of the bacitracin X group.

Studies on the Phosphatide of Aquatic Animals. Part XV. Phosphatidylserine and Phosphatidylethanolamine. (p. *333~335*)

By Kôichi ZAMA and Hisanao IGARASHI

(*Faculty of Fisheries, Hokkaido University, Hakodate*)

A phosphatidylserine- and phosphatidylethanolamine-rich fractions were fractionated from the cephalin of rorqual (*Balaenoptera borealis*) brain by precipitation from $CHCl_3$ solution by increasing concentration of ethanol.

The phosphatidylserine-rich fraction was repeatedly fractionated with $CHCl_3$ -ethanol to yield a phosphatidylserine. A phosphatidylethanolamine was obtained by extracting with warm ethanol containing 5% of $CHCl_3$ from the phosphatidylethanolamine-rich fraction.

Analysis of the phosphatidylserine and the phosphatidylethanolamine gave the following data: P 3.24, 3.14%; N 1.75, 1.69%; serine 11.45, 0%; ethanolamine 0, 7.76%; glycerol 10.35, 10.05%; ash 11.74, 6.49%; iodine no. 76.6, 98.1.

The composition of fatty acids of the phosphatidylserine and the phosphatidylethanolamine was as follows: saturated 29.6, 14.3; monoethylenic 51.1, 60.9; dienoic 2.7, 2.9; trienoic 2.1, 2.5; tetraenoic 1.6, 4.9; pentaenoic 6.3, 8.2; hexaenoic 6.6, 9.2%.

From the corresponding fractions serine, ethanolamine and glycerophosphoric acid were actually separated.

Studies on the Phosphatide of Aquatic Animals. Part XVI. Inositolphosphatide. (p. *336~338*)

By Kôichi ZAMA and Hisanao IGARASHI

(*Faculty of Fisheries, Hokkaido University, Hakodate*)

An inositol-rich fraction was obtained from the rorqual (*Balaenoptera borealis*) brain cephalin by precipitation from $CHCl_3$ solution by adding of ethanol.

This fraction was repeatedly fractionated with $CHCl_3$/methanol to yield an inositolphosphatide, which was analyzed with the following results.

Before dialysis: P 5.16, N 0.18, glycerol 6.98, and ash 20.76%. After dialysis: P 6.31, N 0.23, and glycerol 9.16%.

The component fatty acids were found to consist of saturated 58.2, monoethylenic 29.2, dienoic 1.8, trienoic 1.3, tetraenoic 1.7, pentaenoic 3.5, and hexaenoic 4.3%.

From the inositolphosphatide glycerol, inositol, inositol diphosphate, fatty acids, and phosphoric acid were actually separated.

(*continued from front cover*)

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